



AN INVESTIGATION INTO THE MICROBIAL DEGRADATION OF
BENZYL DIMETHYL HEXADECYLAMMONIUM CHLORIDE USED IN
OILFIELD CHEMICAL FORMULATIONS.

David Etukudo Bassey

Submitted for the Degree of Doctor of Philosophy

Heriot-Watt University

School of Life Sciences

October, 2010

The copyright in this thesis is owned by the author. Any quotation from the thesis or use of any of the information contained in it must acknowledge this thesis as the source of the quotation or information.

ABSTRACT

Benzyltrimethyl Hexadecylammonium Chloride (BDHAC) is a quaternary ammonium compound (QAC) used in corrosion inhibitor formulations. It has both corrosion inhibition and biocidal properties. Studies have suggested that QACs in produced water are often discharged to the marine environment during oil exploration and production. The fate of these compounds on release to the marine environment, especially the ones with a benzyl ring, is not well understood. On reaching the marine environment, QAC adsorb onto sediment and may become bioavailable and toxic to the benthic communities. Therefore bioremediation of sediment could be useful to help preserve the marine environment.

A biodegradation study was carried out using an enrichment culture technique with marine sediment, to identify which marine bacteria degraded BDHAC and subsequent degradation products. Following enrichment in the presence of BDHAC, different strains of bacteria, isolated from marine sediments, were shown to degrade BDHAC in a minimal salts medium. The bacteria identified by 16S rDNA sequencing, were shown to belong to several genera and were identified as *Marine bacterium*, *Bacillus subtilis*, *Bacillus niabensis*, *Sporosarcina* sp., *Thalassospira* sp., *Rhodospirillaceae* and *Staphylococcus equorum*. Investigations revealed that these bacteria were capable of degrading BDHAC when it was present at high concentrations, in the range 2 – 4 mg/ml. In media containing BDHAC, up to 90% of the substance was degraded within 43 days. Quantification of BDHAC after biodegradation experiments were performed by Colorimetric Dye Binding Assay (CDBA) and Electrospray Ionisation Tandem Mass spectrometry (ESI-MS/MS). Preliminary analysis of samples by ESI-MS/MS produced a peak with a parent-daughter ion transition of 136.0→91.0, corresponding to N, N-dimethylbenzylamine. The presence of this potential metabolite suggested the cleavage of the C alkyl-N bond as a step in BDHAC catabolism.

This research will contribute to an understanding of the potential of the isolated strains of bacteria in bioremediating BDHAC contaminated sites. It will also assist operators and regulators in the oil and gas industry to understand the fate of BDHAC in the environment when the compound is used in corrosion inhibitor formulations. This understanding will assist environmental risk assessment of oil production facilities.

DEDICATION

This work is dedicated to my parents Elder and Deaconess Henry Bassey who supported my dreams from the outset.

ACKNOWLEDGEMENTS

My sincere appreciation goes to my supervisors, Dr. Steve Grigson and Dr. Wilf Mitchell for their guidance throughout the entire period of my PhD programme.

I am also grateful to Prof. and Dr. Austin, Dr. Peter Morris, Mrs. Gina Sheperd, Sean McMenamy, Hugh Barras, Margaret Stobie and Alison Forsythe who helped by answering my questions, pointing me to information sources and providing general support during the laboratory phase of my work.

My sincere thanks go to my friends, Joachim, Sharif, John, Elham, Bayani, Mohammed and Elijah for their support and encouragement.

I wish to thank my family for their love, support and prayers. I am indebted to my wife, Grace who has been a tremendous source of encouragement and help.

Finally, I will like to thank AKUTECH, Nigeria for the research grant awarded me.

ACADEMIC REGISTRY Research Thesis Submission

| | | | |
|---|-------------------------|--|-----------------|
| Name: | DAVID ETUKUDO BASSEY | | |
| School/PGI: | SCHOOL OF LIFE SCIENCES | | |
| Version: <i>(i.e. First, Resubmission, Final)</i> | FINAL | Degree Sought (Award and Subject area) | PHD ENVIRONMENT |

Declaration

In accordance with the appropriate regulations I hereby submit my thesis and I declare that:

- 1) the thesis embodies the results of my own work and has been composed by myself
- 2) where appropriate, I have made acknowledgement of the work of others and have made reference to work carried out in collaboration with other persons
- 3) the thesis is the correct version of the thesis for submission and is the same version as any electronic versions submitted*.
- 4) my thesis for the award referred to, deposited in the Heriot-Watt University Library, should be made available for loan or photocopying and be available via the Institutional Repository, subject to such conditions as the Librarian may require
- 5) I understand that as a student of the University I am required to abide by the Regulations of the University and to conform to its discipline.

* *Please note that it is the responsibility of the candidate to ensure that the correct version of the thesis is submitted.*

| | | | |
|-------------------------|--|-------|--|
| Signature of Candidate: | | Date: | |
|-------------------------|--|-------|--|

Submission

| | |
|--|--|
| Submitted By <i>(name in capitals)</i> : | |
| Signature of Individual Submitting: | |
| Date Submitted: | |

For Completion in Academic Registry

| | | | |
|--|--|-------|--|
| Received in the Academic Registry by <i>(name in capitals)</i> : | | | |
| <i>Method of Submission (Handed in to Academic Registry; posted through internal/external mail):</i> | | | |
| <i>E-thesis Submitted (mandatory for final theses from January 2009)</i> | | | |
| Signature: | | Date: | |

TABLE OF CONTENTS

| | Page |
|--|--------------|
| Title Page... .. | i |
| Abstract... .. | ii |
| Dedication... .. | iii |
| Acknowledgments... .. | iv |
| Declaration Statement... .. | v |
| Table of Contents... .. | vi |
| List of Tables | xi |
| List of Figures... .. | xiv |
| List of Abbreviations... .. | xix |
| List of Publications by the Candidate | xxi |
| Introduction to the Research Area... .. | 1 |
| CHAPTER 1... .. | 4 |
| GENERAL INTRODUCTION... .. | 4 |
| 1.1 Drilling and Production Discharges in Marine Environment... .. | 4 |
| 1.2 Nature of Offshore Discharges... .. | 5 |
| 1.2.1 Drilling Waste... .. | 5 |
| 1.2.2 Produced Water... .. | 6 |
| 1.2.3 Types of Production Chemicals... .. | 13 |
| 1.3 Bacteria in the Environment... .. | 20 |
| 1.4 Bacteriocidal Properties of Compounds... .. | 25 |
| 1.5 Microbial Degradation of Quaternary Ammonium Compounds... .. | 26 |
| 1.6 Potential Toxicity of QACs to Marine Organisms... .. | 39 |
| 1.7 Research Outline and Objectives... .. | 41 |

| | |
|--|-----------|
| CHAPTER 2... | 43 |
| PRELIMINARY BIODEGRADATION STUDIES OF BENZYL DIMETHYL- HEXADECYLAMMONIUM CHLORIDE (BDHAC) USING A MARINE BACTERIAL INOCULUM ... | |
| 2.1 Introduction... | 43 |
| 2.2 Aim of the Study... | 43 |
| 2.3 Materials and Methods... | 44 |
| 2.3.1 Glassware Cleaning Procedure... | 44 |
| 2.3.2 Source of BDHAC-utilising Micro-organisms... | 44 |
| 2.3.3 Preparation of Growth Media... | 45 |
| 2.3.4 Preparation of Standards, Test Samples and Controls... | 45 |
| 2.3.5 Isolation of BDHAC Utilising Micro-organisms... | 46 |
| 2.3.6 Characterization of Bacterial Isolates... | 47 |
| 2.3.7 Quantification of Residual BDHAC after Enrichment... | 52 |
| 2.4 Results... | 57 |
| 2.4.1 Isolation of BDHAC-utilising Micro-organisms... | 57 |
| 2.4.2 Characterization of Bacterial Isolates... | 59 |
| 2.4.3 Identification of Bacterial Isolates by 16SrDNA Gene Sequencing... | 60 |
| 2.4.4 Preparation of a Calibration Curve for BDHAC Analysis... | 63 |
| 2.4.5 BDHAC Recovery Analysis... | 64 |
| 2.4.6 Measurement of BDHAC using the CDBA... | 65 |
| 2.4.7 Preparation of Calibration Curve for the Quantification of BDHAC by ESI-MS/MS... | 66 |
| 2.4.8 Quantification of BDHAC by Electrospray Ionization Mass-Spectrometry... | 68 |
| 2.5 Discussion... | 73 |
| 2.6 Conclusion... | 75 |
| CHAPTER 3... | 76 |
| SECTION A | |
| BIODEGRADATION OF BDHAC BY EIGHT STRAINS OF BACTERIA SELECTED FROM PRELIMINARY BIODEGRADATION STUDIES.... | |
| | 76 |

| | |
|--|-----|
| 3.1 Introduction... | 76 |
| 3.2 Aim of the Study... | 76 |
| 3.3 Materials and Methods... | 77 |
| 3.3.1 Preparation of Growth Media... | 77 |
| 3.3.2 BDHAC Degradation Experiment... | 77 |
| 3.3.3 Determination of Optical Density... | 78 |
| 3.3.4 Quantification of the amount of Residual BDHAC by Colorimetric Dye Binding Assay... | 79 |
| 3.3.5 BDHAC Recovery Analysis... | 79 |
| 3.4 Results... | 80 |
| 3.4.1 Optical Density Values Showing Growth of Bacteria at Different Concentrations of BDHAC... | 80 |
| 3.4.2 Preparation of Calibration Curve... | 92 |
| 3.4.3 BDHAC Recovery Analysis... | 93 |
| 3.4.4 Quantification of Residual BDHAC by Colorimetric Dye Binding Analysis (CDBA)... | 95 |
| 3.5 Discussion... | 103 |
| 3.6 Conclusion... | 104 |

SECTION B

| | |
|--|-----|
| BIODEGRADATION OF BDHAC BY FOUR STRAINS OF BACTERIA... | 105 |
| 3.7 Introduction... | 105 |
| 3.8 Aim of the Study... | 105 |
| 3.9 Materials and Methods... | 106 |
| 3.10 Results..... | 108 |
| 3.11 Discussion... | 118 |
| 3.12 Conclusion... | 119 |

SECTION C

| | |
|--|-----|
| BIODEGRADATION OF BDHAC BY THE FOUR STRAINS OF BACTERIA INVESTIGATED IN SECTION B AND INCUBATED IN MARINE BROTH MEDIA... | 120 |
| 3.13 Introduction... | 120 |

| | | | | | | | | |
|--|-----|-----|-----|-----|-----|-----|-----|------------|
| 3.14 Aim of the Study... | ... | ... | ... | ... | ... | ... | ... | 120 |
| 3.15 Materials and Methods | ... | ... | ... | ... | ... | ... | ... | 120 |
| 3.16 Results..... | ... | ... | ... | ... | ... | ... | ... | 124 |
| 3.17 Discussion... | ... | ... | ... | ... | ... | ... | ... | 140 |
| 3.18 Conclusion... | ... | ... | ... | ... | ... | ... | ... | 140 |
| CHAPTER 4... | ... | ... | ... | ... | ... | ... | ... | 141 |
| IDENTIFICATION OF PRODUCTS FROM BACTERIAL DEGRADATION | | | | | | | | |
| OF BDHAC ... | ... | ... | ... | ... | ... | ... | ... | 141 |
| 4.1 Introduction... | ... | ... | ... | ... | ... | ... | ... | 141 |
| 4.2 Aim of the Study... | ... | ... | ... | ... | ... | ... | ... | 141 |
| 4.3 Materials and Methods... | ... | ... | ... | ... | ... | ... | ... | 142 |
| 4.4 Results... | ... | ... | ... | ... | ... | ... | ... | 142 |
| 4.5 Discussion... | ... | ... | ... | ... | ... | ... | ... | 146 |
| 4.6 Conclusion... | ... | ... | ... | ... | ... | ... | ... | 149 |
| CHAPTER 5... | ... | ... | ... | ... | ... | ... | ... | 150 |
| DISCUSSION OF RESEARCH FINDINGS... | | | | | | | | |
| 5.1 Discussion... | ... | ... | ... | ... | ... | ... | ... | 150 |
| 5.1.1 Biodegradation and Bioremediation... | ... | ... | ... | ... | ... | ... | ... | 150 |
| 5.1.2 Bacterial Degradation of Quaternary Ammonium Compounds... | ... | ... | ... | ... | ... | ... | ... | 151 |
| 5.1.3 Identification of Products from BDHAC Biodegradation... | ... | ... | ... | ... | ... | ... | ... | 156 |
| 5.1.4 Application of the Research Findings in Bioremediation ... | ... | ... | ... | ... | ... | ... | ... | 159 |
| 5.2 Conclusion... | ... | ... | ... | ... | ... | ... | ... | 161 |
| 5.3 Future Work... | ... | ... | ... | ... | ... | ... | ... | 162 |
| REFERENCES... | ... | ... | ... | ... | ... | ... | ... | 165 |
| APPENDIX... | ... | ... | ... | ... | ... | ... | ... | 188 |
| APPENDIX A... | ... | ... | ... | ... | ... | ... | ... | 188 |
| A.1: BDHAC RECOVERY ANALYSIS (CHAPTER 2) ... | ... | ... | ... | ... | ... | ... | ... | 188 |

| | |
|---|-----|
| A.2: BDHAC RECOVERY ANALYSIS (CHAPTER 3 <i>Section A</i>) | 189 |
| A.3: BDHAC RECOVERY ANALYSIS (CHAPTER 3 <i>Section B</i>) | 192 |
| A.4: BDHAC RECOVERY ANALYSIS (CHAPTER 3 <i>Section C</i>) | 194 |
| APPENDIX B... .. | 196 |
| B.1 QUANTIFICATION OF RESIDUAL BDHAC BY COLORIMETRIC DYE BINDING ASSAY (CDBA) (CHAPTER 2).. .. | 196 |
| B.2 QUANTIFICATION OF RESIDUAL BDHAC BY ELECTROSPRAY IONISATION MASS SPECTROMETRY (ESI-MS/MS) (CHAPTER 2)... .. | 201 |
| APPENDIX C... .. | 204 |
| C.1: QUANTIFICATION OF RESIDUAL BDHAC AFTER BIODEGRADATION EXPERIMENT BY COLORIMETRIC DYE BINDING ASSAY (CDBA) (CHAPTER 3 <i>Section A</i>)... .. | 204 |
| APPENDIX D... .. | 210 |
| D.1 QUANTIFICATION OF RESIDUAL BDHAC AFTER BIODEGRADATION EXPERIMENT BY COLORIMETRIC DYE BINDING ASSAY (CDBA) (CHAPTER 3 <i>Section B</i>)... .. | 210 |
| APPENDIX E... .. | 214 |
| E.1 QUANTIFICATION OF RESIDUAL BDHAC BY COLORIMETRIC DYE BINDING ASSAY (CDBA) AFTER BIODEGRADATION EXPERIMENT (CHAPTER 3 <i>Section C</i>)... .. | 214 |
| E.2 TABLES SHOWING OD ₆₅₀ , ABSORBANCE AND AMOUNT OF RESIDUAL BDHAC FROM GROWTH AND BIODEGRADATION EXPERIMENTS IN SECTION C OF CHAPTER 3... .. | 232 |

LISTS OF TABLES

| | Page |
|--|------|
| Table 1.1 Produced Water Discharges, North Sea... .. | 7 |
| Table 1.2 Mean Metal Concentrations (mg/kg dry wt) in Commercial Fishes and Crabs, Gulf of Cambay, India in 2006.... .. | 11 |
| Table 1.3 Aquatic Toxicity Data for QACs..... | 40 |
| Table 2.1 Viable Colony Counts and CFU/ml Values from Samples during 43 days Incubation... .. | 58 |
| Table 2.2 Physiological Characteristics and Tests on Bacterial Strains... .. | 59 |
| Table 2.3 16S-rDNA Analysis on Bacterial Isolates... .. | 62 |
| Table 2.4 Relationship between BDHAC Concentration and Absorbance ... | 63 |
| Table 2.5 Recovery of BDHAC from Sample Solutions... .. | 65 |
| Table 2.6 Quantification of BDHAC in the Test and Control Samples by CDBA. | 66 |
| Table 2.7 Quantification of BDHAC in the Test and Control Samples by ESI-MS/MS... .. | 71 |
| Table 3.1 Concentration and Absorbance Values for the Plotting of a Calibration Curve... .. | 93 |
| Table 3.2 BDHAC Amounts Added, Concentration and Residual Amounts after Biodegradation in Samples with <i>Micrococcus luteus</i> E from Day 28 of Incubation... .. | 95 |
| Table 3.3 BDHAC Amounts Added, Concentration and Residual Amounts after Biodegradation in Samples with <i>Bacillus niabensis</i> from Day 28 of Incubation... .. | 96 |
| Table 3.4 BDHAC Amounts Added, Concentration and Residual Amounts after Biodegradation in Samples with <i>Bacillus subtilis</i> from Day 28 of Incubation... .. | 97 |

| | |
|---|-----|
| Table 3.5 BDHAC Amounts Added, Concentration, and Residual Amounts after Biodegradation in Samples with <i>Rhodospirillaceae</i> from Day 28 of Incubation... | 98 |
| Table 3.6 BDHAC Amounts Added, Concentration, Absorbance and Residual Amounts after Biodegradation in Samples with <i>Thalassospira sp.</i> from Day 28 of Incubation... | 99 |
| Table 3.7 BDHAC Amounts Added, Concentration, Absorbance and Residual Amounts after Biodegradation in Samples with <i>Micrococcus luteus</i> C from Day 28 of Incubation... | 100 |
| Table 3.8 BDHAC Amounts Added, Concentration, Absorbance and Residual Amounts after Biodegradation in Samples with <i>Staphylococcus equorum</i> from Day 28 of Incubation..... | 101 |
| Table 3.9 BDHAC Amounts Added, Concentration, Absorbance and Residual Amounts after Biodegradation in Samples with <i>Sporosarcina sp.</i> from Day 28 of Incubation... | 102 |
| Table 3.10 Physiological Characteristics and Biochemical Tests on Bacterial Strains | 109 |
| Table 3.11 Percent BDHAC Recovery Values from Experiment... | 112 |
| Table 3.12 Concentration and Absorbance Values Used to Construct a Calibration Curve... | 113 |
| Table 3.13 Concentration, Volume and Absorbance of Calibration Standards... | 134 |
| E.2: Table 1. Optical Density Values Showing the Growth of <i>Bacillus niabensis</i> at Different Concentrations of BDHAC... | 232 |
| E2: Table 2. Optical Density Values Showing the Growth of <i>Bacillus subtilis</i> at Different Concentrations of BDHAC... | 232 |
| E2: Table 3 Optical Density Values Showing the Growth of <i>Sporosarcina sp.</i> at Different Concentrations of BDHAC... | 233 |
| E2: Table 4. Optical Density Values Showing the Growth of <i>Thalassospira sp.</i> at Different Concentrations of BDHAC... | 233 |
| E2: Table 5. Absorbance Values at Different Concentrations of BDHAC with <i>Bacillus niabensis</i> ... | 234 |

| | |
|--|-----|
| E2: Table 6. Amount of Residual BDHAC after BDHAC Degradation with <i>Bacillus niabensis</i> ... | 234 |
| E2: Table 7. Absorbance Values at Different Concentrations of BDHAC with <i>Bacillus subtilis</i> ... | 235 |
| E2: Table 8. Amount of Residual BDHAC after BDHAC Degradation with <i>Bacillus subtilis</i> ... | 235 |
| E2: Table 9. Absorbance Values at Different Concentrations of BDHAC with <i>Sporosarcina sp.</i> ... | 236 |
| E2: Table 10. Amount of Residual BDHAC after BDHAC Degradation with <i>Sporosarcina sp.</i> ... | 236 |
| E2: Table 11. Absorbance Values at Different Concentrations of BDHAC with <i>Thallossospira sp.</i> ... | 237 |
| E2: Table 12. Amount of Residual BDHAC after BDHAC Degradation with <i>Thallossospira sp.</i> ... | 237 |

LIST OF FIGURES

| | Page |
|--|------|
| Figure 1.1 Schematic Presentation of the Research Area... .. | 3 |
| Figure 1.2 Structure of Benzyl Dimethylhexadecylammonium Chloride [BDHAC]..... | 18 |
| Figure 1.3 3D Structure of Benzyl Dimethylhexadecylammonium Chloride [BDHAC]... | 19 |
| Figure 1.4 Cross-section through the wall of a typical Gram-positive bacterium. Peptidoglycan is the main constituent of the wall and is thicker than in the case of Gram-negative species. Source: (Wilson et al. 2002) ... | 23 |
| Figure 1.5 Cross-section through the wall of a typical Gram-negative bacterium. The wall is multilayered and consist a thinner layer of peptidoglycan than in the case of Gram-positive species. Source: (Wilson et al. 2002). ... | 23 |
| Figure 1.6 Possible Mechanisms of Degradation of QACs by Bacteria... .. | 28 |
| Figure 1.7 Proposed Biodegradation Pathway of Hexadecyltrimethylammonium Chloride by <i>Pseudomonas</i> Strain B1. (1) Monooxygenase; (2) Alkanal Dehydrogenase van Ginkel, et al., (1992).... | 30 |
| Figure 1.8 Possible Degradation Pathway of DDAC by <i>P. fluorescens</i> TN4. Nishihara, et al., (2000).... | 31 |
| Figure 1.9 Proposed Pathway for Benzyl dimethylalkylammonium Chloride Biodegradation by <i>Aeromonas hydrophila</i> sp. K (Patrauchan and Oriol, 2003)... | 33 |
| Figure 1.10 Proposed Pathways of <i>n</i> -dodecyltrimethylammonium Metabolism by <i>Pseudomonas</i> sp. strain 7-6. I, <i>n</i> -dodecyltrimethylammonium; II, <i>n</i> -dodecanal; III, lauric acid; IV, trimethylamine; V, dimethylamine; VI, <i>n</i> -dodecyldimethylamine (Takenaka, et al., 2007)... | 36 |
| Figure 2.1 Schematic of an ESI-MS Source... .. | 55 |
| Figure 2.2 Identification of Bacterial Isolates... .. | 61 |

| | |
|---|----|
| Figure 2.3 Calibration Curve for Colorimetric Dye Binding Assay (CDBA)... | 64 |
| Figure 2.4 Calibration Curve for BDHAC Analysis by ESI-MS/MS... .. | 67 |
| Figure 2.5 Chromatogram for the Quantification of BDHAC in Control Sample | 68 |
| Figure 2.6 Chromatogram for the Quantification of BDHAC in Sample DP1... | 69 |
| Figure 2.7 Chromatogram for the Quantification of BDHAC in sample DP2... | 69 |
| Figure 2.8 Chromatogram for the Quantification of BDHAC in sample DP3... | 70 |
| Figure 2.9 Chromatogram for the Quantification of BDHAC in sample DP4... | 70 |
| Figure 2.10 Chromatogram for the Quantification of BDHAC in sample DP5... | 71 |
| Figure 3.1 Growth of <i>Micrococcus luteus</i> E at Different BDHAC Concentrations | 81 |
| Figure 3.2 Optical Density as a Function of BDHAC Concentration for <i>Micrococcus luteus</i> E | 81 |
| Figure 3.3 Growth of <i>Bacillus niabensis</i> at Different BDHAC Concentrations | 82 |
| Figure 3.4 Optical Density as a Function of BDHAC Concentration for <i>Bacillus niabensis</i> | 83 |
| Figure 3.5 Growth of <i>Bacillus subtilis</i> F at Different BDHAC Concentrations | 84 |
| Figure 3.6 Optical Density as a Function of BDHAC Concentration for <i>Bacillus subtilis</i> | 84 |
| Figure 3.7 Growth of <i>Rhodospirillaceae</i> at Different BDHAC Concentrations | 85 |
| Figure 3.8 Optical Density as a Function of BDHAC Concentration for <i>Rhodospirillaceae</i> | 86 |
| Figure 3.9 Growth of <i>Thalassospira sp.</i> at Different BDHAC Concentrations. | 87 |
| Figure 3.10 Optical Density as a Function of BDHAC Concentration for <i>Thalassospira sp.</i> | 87 |
| Figure 3.11 Growth of <i>Micrococcus luteus</i> C at Different BDHAC Concentrations... .. | 88 |
| Figure 3.12 Optical Density as a Function of BDHAC Concentration for <i>Micrococcus luteus</i> C... .. | 89 |

| | |
|--|-----|
| Figure 3.13 Growth of <i>Staphylococcus equorum</i> at Different BDHAC Concentrations... | 90 |
| Figure 3.14 Optical Density as a Function of BDHAC Concentration for <i>Staphylococcus equorum</i> ... | 90 |
| Figure 3.15 Growth of <i>Sporosarcina sp.</i> at Different BDHAC Concentrations | 91 |
| Figure 3.16 Optical Density as a Function of BDHAC Concentration for <i>Sporosarcina sp.</i> ... | 92 |
| Figure 3.17 Calibration Curve for Dye Binding Assay... | 93 |
| Figure 3.18 Percent Recovery from Flasks Containing Different Concentrations of BDHAC ... | 94 |
| Figure 3.19 Growth of <i>Bacillus niabensis</i> in Medium Containing 2 mg/ml BDHAC... | 109 |
| Figure 3.20 Growth of <i>Bacillus subtilis</i> in Medium Containing 4 mg/ml BDHAC | 110 |
| Figure 3.21 Growth of <i>Sporosarcina sp.</i> in Medium Containing 2 mg/ml BDHAC | 111 |
| Figure 3.22 Growth of <i>Thalassospira sp.</i> in Medium Containing 4 mg/ml BDHAC... | 111 |
| Figure 3.23 Calibration Curve for Calorimetric Dye Binding Assay... | 114 |
| Figure 3.24 Degradation of BDHAC by <i>Bacillus niabensis</i> . as Determined by CDBA... | 114 |
| Figure 3.25 Degradation of BDHAC by <i>Bacillus subtilis</i> . as Determined by CDBA... | 115 |
| Figure 3.26 Degradation of BDHAC by <i>Sporosarcina sp.</i> as Determined by CDBA... | 116 |
| Figure 3.27 Degradation of BDHAC by <i>Thalassospira sp.</i> as Determined by CDBA... | 117 |
| Figure 3.28 Graph Showing OD ₆₅₀ and CFU/ml Values for <i>Bacillus niabensis</i> in the Presence and Absence of 2 % Methanol..... | 125 |
| Figure 3.29 Graph Showing OD ₆₅₀ and CFU/ml Values for <i>Sporosarcina sp.</i> in the Presence and Absence of 2 % Methanol..... | 126 |

| | |
|---|-----|
| Figure 3.30 Graph Showing OD ₆₅₀ and CFU/ml Values for <i>Bacillus subtilis</i> in the Presence and Absence of 2 % Methanol.... | 127 |
| Figure 3.31 Graph Showing OD ₆₅₀ and CFU/ml Values for <i>Thalassospira sp.</i> in the Presence and Absence of 2 % Methanol ... | 128 |
| Figure 3.32 Relationship of Colony Forming Unit (CFU/ml x10 ⁷) and Optical Density (OD ₆₅₀) for the different Strains of Bacteria... | 129 |
| Figure 3.33 Relationship of Colony Forming Unit (CFU/ml x10 ⁷) and Optical Density (OD ₆₅₀) for the different Strains of Bacteria in the presence of 2ml of Methanol... | 130 |
| Figure 3.34 Growth of <i>Bacillus niabensis</i> (OD ₆₅₀) at Different Concentrations of BDHAC during 120 hours of Incubation... | 131 |
| Figure 3.35 Growth of <i>Bacillus subtilis</i> (OD ₆₅₀) at Different Concentrations of BDHAC in 120 Hours of Incubation... | 132 |
| Figure 3.36 Growth of <i>Sporosarcina sp.</i> (OD ₆₅₀) at Different Concentrations of BDHAC During 120 hours of Incubation... | 132 |
| Figure 3.37 Growth of <i>Thalassospira sp.</i> (OD ₆₅₀) at Different Concentrations of BDHAC during 120 hours of Incubation... | 133 |
| Figure 3.38 Calibration Curve for CDBA... | 134 |
| Figure 3.39 Percent Recovery of BDHAC at Different Concentrations... | 135 |
| Figure 3.40 Residual Amount of BDHAC from cultures of <i>Bacillus niabensis</i> Incubated for 120 hours... | 136 |
| Figure 3.41 Residual Amount of BDHAC from Cultures of <i>Bacillus subtilis</i> Incubated for 120 hours... | 137 |
| Figure 3.42 Residual Amount of BDHAC from Cultures of <i>Sporosarcina sp.</i> Incubated for 120 hours... | 138 |
| Figure 3.43 Residual Amount of BDHAC from Cultures of <i>Thalassospira sp.</i> Incubated for 120 hours... | 139 |
| Figure 4.1 ESI-MS Spectra and Chromatogram Showing Pure Standard of Benzyldimethylamine and that Formed after Biodegradation of BDHAC by the Strains of Bacteria... | 143 |

| | | | | | | |
|--|-----|-----|-----|-----|-----|-----|
| Figure 4.2 Proposed Degradation Pathway of BDHAC Showing the Formation of Benzyldimethylamine (BDAM)... | ... | ... | ... | ... | ... | 144 |
| Figure 4.3 ESI-MS Spectra Showing Pure Standards of (a) N-Benzylmethylamine and (b) Benzylamine that were also Tested... | ... | ... | ... | ... | ... | 145 |
| Figure 4.4 ESI-MS Spectra Showing Pure Standards of (e) Benzaldehyde and (f) Benzoic Acid that were also Tested... | ... | ... | ... | ... | ... | 146 |

LIST OF ABBREVIATIONS

| | |
|------------|--|
| OECD | Organisation for Economic Co-operation and Development |
| EEC | European Economic Community |
| DDA | Dodecyldimethylammonium chloride |
| TMA | Trimethylamine |
| DMA | Dimethylamine |
| BAC | Benzyldimethylalkylammonium chloride |
| GC-MS | Gas Chromatography–Mass Spectrometry |
| DTAC | DodecylTrimethylAmmonium Chloride |
| QAC | Quaternary Ammonium Compounds |
| TMAC | Trimethyl Ammonium Chloride |
| PEC | Predicted Environmental Concentration |
| PNEC | Predicted No-Effect Concentration |
| BDHAC | BenzyldimethylHexadecylAmmonium Chloride |
| PCR | Polymeric Chain Reaction |
| EDTA | Ethylene Diamine Tetra Acetic acid |
| BLAST | Basic Local Alignment Search Tool |
| CDBA | Colorimetric Dye Binding Assay |
| ESI-MS/MS. | Electrospray Ionisation Tandem Mass Spectrometry |
| MRM | Multiple Reaction Monitoring |
| NCBI | National Center for Biotechnology Information |
| DCM | Dichloromethane |
| OD | Optical Density |

| | |
|------------------|--|
| CFU | Colony Forming Unit |
| ANOVA | Analysis of Variance |
| BMAM | Benzylmethanamine |
| BDAM | Benzyltrimethylammonium chloride |
| PAH | Polycyclic Aromatic Hydrocarbon |
| OSPAR | Oslo and Paris Commission |
| UKOOA | United Kingdom Offshore Operators Association |
| BTEX | Benzene,Toluene,Ethylbenzene,Xylene |
| SD | Standard Deviation |
| E&P | Exploration and Production |
| EC ₅₀ | The concentration of chemical that produces a specified effect in 50% of the test organism |
| TMAC | Trimethyl ammonium chloride |
| DADMAC | Dialkyl dimethyl ammonium chloride |
| BDMAC | Dodecylbenzyltrimethyl ammonium chloride |
| DTDMAC | Ditoluene dimethyl ammonium chloride |
| DEEDMAC | Diethylester dimethylammonium chloride |

LIST OF PUBLICATIONS BY THE CANDIDATE

Bassey, D. E, Grigson, S.J.W, and Mitchell, W.J (2009). Microbial Degradation of Benzyldimethyl Hexadecylammonium Chloride Used in Oilfield Chemical Formulations. A paper presented at the 2009 SPE Americas E&P Environmental and Safety Conference, held in San Antonio, Texas, USA, 23-25th March 2009.

Bassey, D. E and Grigson, S.J.W. (2010). Degradation of Benzyldimethyl Hexadecylammonium Chloride by *Bacillus niabensis* and *Thalassospira* sp. Isolated from Marine Sediments. Journal of Toxicology and Environmental Chemistry 1-13, iFirst

INTRODUCTION TO THE RESEARCH AREA

Oil and gas production is a source of pollutants to the marine environment through the discharge of waste (produced) water. Figure 1.1 shows the nature of these pollutants and the selection process for the pollutant under investigation. This is expressed as a series of levels (1-7), each of which is described below.

Level 1: Oil exploration and production is often associated with the discharge of waste to the environment. The discharged waste includes drilling fluids, produced water and drill cuttings. This research will focus on the discharge of produced water to the marine environment.

Level 2: Produced water contains a range of pollutants. These include dissolved oil, trace metals and oilfield chemicals. This research will investigate residual process chemicals. These are of concern due to their often complex chemistries and their potential environmental impacts when discharged along with produced water.

Level 3: A wide range of oilfield chemicals are used by the oil and gas industry to mitigate operational problems. This study will investigate corrosion inhibitors. These have been selected owing to their wide applications in oil and gas exploration and production processes and environmental concerns over their discharge to the marine environment.

Levels 4 and 5: Ranges of nitrogen-containing chemistries are used to combat corrosion in offshore facilities. Quaternary ammonium compounds (QACs) have been selected for investigation. QACs are cationic surfactants which are molecules with a hydrophobic hydrocarbon chain linked to a positively charged nitrogen atom. These compounds are known to produce adverse environmental affects on marine organisms and assays are available for their analysis.

Level 6: The specific chemical chosen for investigation is benzyldimethylhexadecyl ammonium chloride (BDHAC). This compound is known to be widely used in oilfield chemical formulations and is commercially available in a pure form.

Level 7: The degradation of BDHAC by bacteria isolated from marine sediments was investigated to obtain information on: the types and species of bacteria that could degrade the compound; relative degradation rates; and potential products of degradation. The ability of bacteria to degrade high concentrations of BDHAC for bioremedial purposes was also investigated.

A schematic presentation of the research area is shown in Figure 1.1. The thick arrows in the middle of the diagram show the focus of the research investigation.

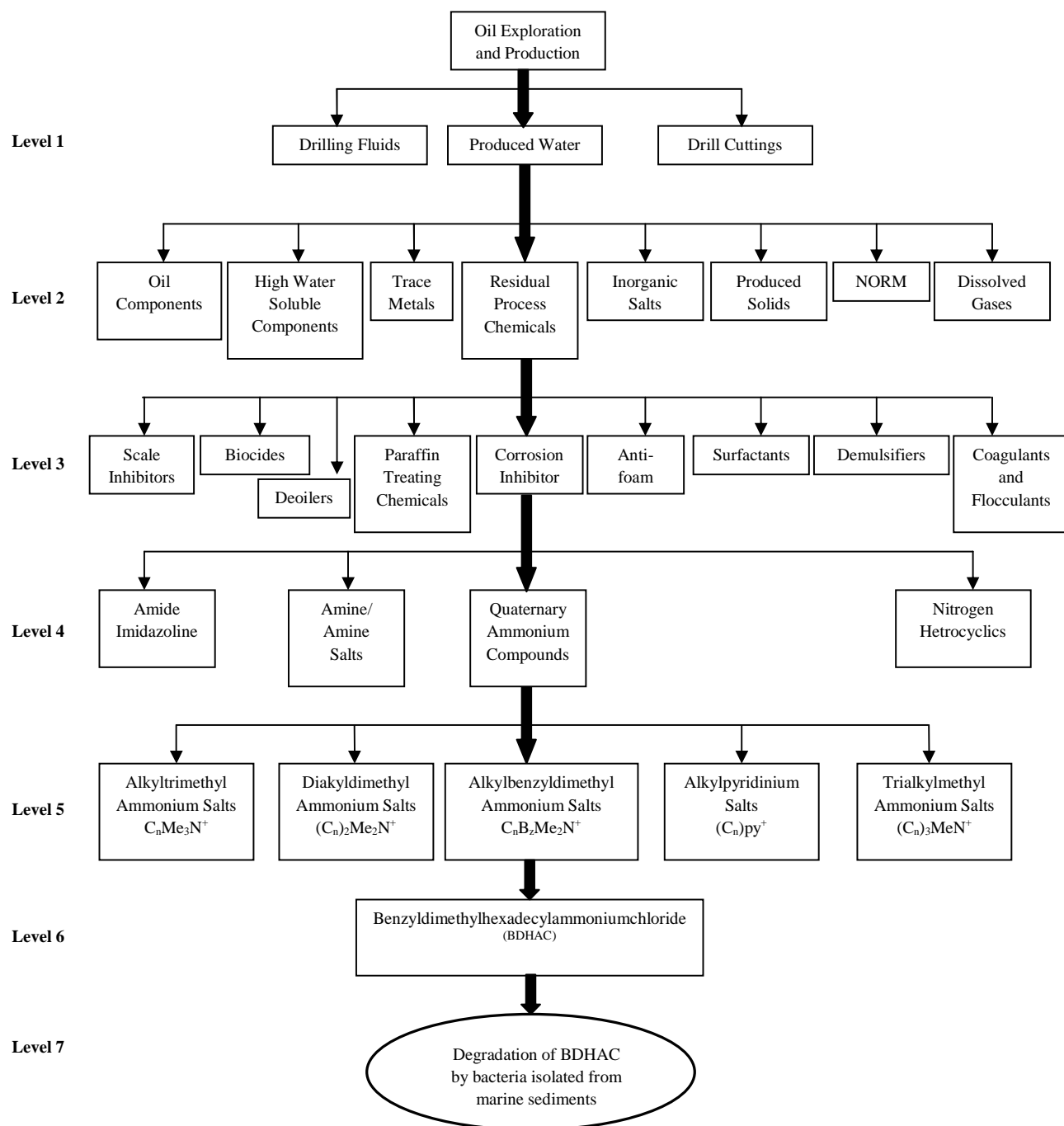


Figure 1.1 Schematic Presentation of the Research Area.

CHAPTER 1

GENERAL INTRODUCTION

This section gives an overview of the research area relevant to the oil industry. It highlights the research problem and its importance. Aspects of the existing literature are discussed with the aim of revealing the research gaps which this research addresses. Furthermore, the general methodology used to address the problems identified is reviewed.

1.1 Drilling and Production Discharges in the Marine Environment

Crude oil is a naturally occurring, flammable liquid found in rock formations in the Earth and consists of a mixture of hydrocarbons of various molecular weights, and other organic compounds. It can be yellow to black in color and with an unpleasant odour. Prior to oil production, rocks and soil samples from the ground in specific and targeted areas are examined and if the results of the rock studies at the site show evidence of the availability of crude oil, drilling will commence at the marked site. During drilling, an oil well is created by drilling a hole into the earth with an oil rig. A steel pipe (casing) is then placed in the hole to provide structural integrity to the newly drilled wellbore. Holes are then made in the base of the well to enable oil to pass into the bore. Finally a collection of valves called a production tree is fitted to the top, the valves regulating pressure and control fluid flow. From the outlet valve of the production tree, the flow can be connected to a network of pipelines and tanks for supply of the petroleum product to refineries and natural gas compressor stations, or oil export terminals. Soon after offshore exploration and production (E&P) operations began in the North Sea, concerns arose about the potential environmental impact of exploration and production activities. The first regulations were developed by the Oslo and Paris (OSPAR) Convention to control discharges from production platforms and drilling rigs. Over the years treaties, laws and regulations have been promulgated so that drilling and production discharges are strictly controlled by a system of limits in the North Sea. A complicating factor in the early stages

of offshore environmental standards was that their development coincided with rapid changes in the technology used in offshore operations. However, the objective of the rules and regulations has always been, and still is, to allow offshore exploration and production to occur while minimizing any associated environmental impacts (Orszulik, 2008).

In order to develop effective regulations and the technology required to ensure that the discharges meet the limits, it is necessary to understand both the nature and volumes of the discharges and the sensitivities of the receiving environment. Offshore operations may be in international waters, national waters, or in waters under local jurisdiction. In some cases this can mean that more than one regulatory body may be involved. The characteristics of the water bodies receiving discharged wastes vary widely. Some of the important factors in determining sensitivity to the impact of discharges are: the chemical and physical characteristics of the waste, water depth, distance from the shore, typical wind and wave forces in the area and the presence of sensitive marine communities (Orszulik, 2008).

The nature of wastes discharged is affected by several factors including regulations, operator policies and practices. The wastes generated by oil and gas exploration and production operations can be divided into two broad categories: those from oil and gas operations themselves and those due to support activities. The major wastes by volume, from drilling and production operations include: produced water, water based drilling mud, drill cuttings and waste that requires handling during site abandonment (Orszulik, 2008).

1.2 Nature of Offshore Discharges

Offshore discharges are heterogeneous in nature. They represent a mixture of components that result from offshore drilling processes. Examples of offshore discharges are discussed below.

1.2.1 Drilling Waste

Drilling waste includes drilling fluids (or mud) and the formation fragments (cuttings) removed in the drilling process. Drilling fluids are suspensions of solids and other materials

in a liquid base. The composition and properties of drilling fluids are determined by their functions. Three of the primary functions drilling muds perform are: lubricating and cooling the drill bit, maintaining downhole hydrostatic pressure and cleaning out the hole by bringing cuttings to the surface. Drilling fluids fall into one of three classes based on the fluid comprising the mud: water based mud, oil based mud and synthetic based mud. More information on the nature of drilling muds and cuttings discharges can be found in Reis (1996).

1.2.2 Produced Water

Produced water is the largest volume waste stream in the oil exploration and production industry. As fields mature, the volume of produced water can be greater in magnitude than the volume of oil produced. The chemical composition of produced water gives rise to its environmental impacts. Produced water includes the water native to the producing formation, water injected into the formation to increase reservoir pressure and to sweep oil from the formation, and various well treatment solutions and chemicals added during production and the oil/water separation process on the platform (Stephenson, 1992). A mixture of oil, gas, and water, is received from a hydrocarbon producing well and can be separated by means of a separator in the form of a vessel divided into separation chambers and provided with a dynamic separator device which increases the droplet size of the oil and water of the mixture by flowing the mixture spirally, linearly, and thereafter into the first segment of the separation chambers. Here the mixture impacts against the walls of the separating chamber and the gas partially separates from the resultant oil and water mixture. The partially separated gas migrates to the upper end of the first chamber and is further stripped before flowing out via a gas outlet. The residual liquid mixture descends into a lower chamber where it separates into a water phase and an oil phase and the oil is subsequently recovered from the oil phase. (Reis, 1996).

The volume of produced water varies over the life cycle of an oilfield, typically increasing over time. Table 1.1 shows produced water volumes discharged from oil and gas installations in the United Kingdom sector of the North Sea between 1996 and 1998.

Table 1.1 Produced Water Discharges, North Sea.

| Year | Number of installations | Water quantity (millions of tonnes) | Oil levels (ppm) | Oil quantity (tonnes) |
|------|-------------------------|-------------------------------------|------------------|-----------------------|
| 1996 | 59 | 210 | 27 | 5,706 |
| 1997 | 64 | 234 | 25 | 5,764 |
| 1998 | 67 | 253 | 22 | 5,690 |

Source: UKOOA, 1998

Produced water discharged to the environment has potential environmental impacts. Environmental impact has to do with a variety of effects of discharges on the receiving environment. The effects can range from minor changes in the chemical composition of receiving water to major changes in the chemical, physical and biological nature of water columns, sediments, flora and fauna. Consequently, the acute and chronic toxic effects (short and long -term toxic effects) of produced water discharges in both the water and sediment phase after discharge are important (Brendehaug et al., 1992). A constituent may be toxic, but unless ingested or absorbed by an organism at levels or concentrations above a sensitivity threshold, effects are not likely to occur. The main groups of produced water components potentially responsible for its acute toxicity are: dissolved and dispersed oil and other organics, heavy metals, major ions responsible for salinity and osmotic properties of water, and residual production chemicals. Chemicals like corrosion inhibitors, scale inhibitors, emulsion breakers, flocculants, antifoams and biocides are all used during offshore production. Their influence on produced water quality can affect several factors, including oil/water partition coefficient, acute and chronic toxicity, bioavailability and biodegradability (Brendehaug et al., 1992).

Several studies have indicated that acute toxicity of produced water to marine organisms is generally low, except possibly in the mixing zone. This is chiefly due to rapid dilution and biodegradation of the aromatic and phenol fractions. Studies which have investigated the acute toxicity of produced water to marine species also report planktonic

species to be among the most sensitive (Brendehaug et al., 1992; E&P Forum, 1994; Henriquez, 1992; Somerville et al., 1987; Stromgren et al., 1995). More information on offshore produced water discharges can be found in Ray and Engelhardt (1992).

Produced Water Composition: The basic components of produced water can be grouped into the following categories:

- Dissolved and dispersed oil components derived from the reservoir
- Other highly water-soluble components
- Trace metals
- Inorganic salts
- Produced solids
- Dissolved gasses including O₂, CO₂ and H₂S
- Residual process chemicals

Oil Components: This is the term applied to organic materials dispersed or dissolved in produced water at the time it is discharged to the aquatic environment. The oil content of produced water is the most thoroughly monitored and regulated parameter. Oil is the term applied to organic material that is dispersed or dissolved in produced water during discharge (Stephenson, 1992). Oil is an important component of any discharge since it can create potentially toxic effects near points of discharge. Dispersed oil consists of small droplets suspended in the aqueous phase. When oil from produced water comes in contact with the ocean floor, contamination and accumulation of oil by ocean sediments may occur. This can alter the state of the benthic community. The amount of dispersed oil in a produced water stream will vary depending on oil density, interfacial tension (the surface free energy that exists between two immiscible liquid phases, such as oil and water) between oil and water phases, type and efficiency of chemical treatment type, and size and efficiency of the physical separation equipment (Ali et al., 1999). Soluble organics and treatment chemicals contained in produced water decrease the interfacial tension between oil and water (Bansal and Caudle, 1999). Dispersed oils can rise to the surface following discharge and spread, resulting in sheening and increased biological oxygen demand near the point of discharge (Stephenson 1992).

Other Highly Water-Soluble Components: These include dissolved hydrocarbons such as benzene, toluene and xylene, and substantial amounts of non-hydrocarbon organic matter, largely salts of acetic, propionic and butyric acids, which are largely biodegradable. The largest groups of organic components found in produced water are low molecular weight aromatic compounds, carboxylic acids and phenols. Solubility of organic compounds depends on temperature and pH (McFarlane et al., 2002). Hydrocarbons that are naturally present in produced water include: organic acids, polycyclic aromatic hydrocarbons (PAHs) (these are also found in crude oil and those with more than three rings are not very water soluble), phenols, and volatiles. These hydrocarbons are likely to contribute to the toxicity of produced water and the toxicities of these naturally occurring hydrocarbons have been reported to be additive (Glickman 1998).

Soluble organics in produced water are often discharged to the ocean or re-injected at onshore or offshore locations basically because they cannot be removed from produced water. The concentration of organic compounds in produced water increases as the molecular weight of the compound decreases. Lighter molecular weight compounds such as benzene, toluene, ethylbenzene and xylene (BTEX) are less influenced by the efficiency of the oil/water separation process than the higher molecular weight Polycyclic Aromatic Hydrocarbons (PAHs) (Utvik, 2003). Volatile hydrocarbons are present naturally in produced water. Concentrations of volatile hydrocarbons are usually higher in produced water from gas-condensate-producing platforms than in produced water from oil-producing platforms (Utvik, 2003). Natural gas condensate (NGC) can be defined as a low-density mixture of hydrocarbon liquids that are present as gaseous components in the raw natural gas produced from many natural gas fields. NGC condenses out of the raw gas when the temperature is reduced to below the hydrocarbon dew point temperature of the raw gas. It is also referred to as condensate or gas condensate.

Some organic components are very soluble in produced water. They consist of low molecular weight (C2-C5) carboxylic acids (fatty acids), ketones, alcohols, propionic acid, acetone and methanol (Ali et al., 1999). Medium to higher molecular weight hydrocarbons (C6-C15) are the partially soluble components in produced water. They are soluble at low concentrations, but are not as soluble as lower molecular weight hydrocarbons. They are also discharged to the ocean because they cannot be easily removed from produced water.

They include aliphatic and aromatic carboxylic acids, phenols and aliphatic and aromatic hydrocarbons. Aromatic hydrocarbons consist of carbon and hydrogen in a benzene-like cyclic system (Danish EPA, 2003). The detailed chemistry of aromatic compounds is described in Lee et al., (1981). PAHs are hydrocarbons with several cyclic rings and are formed naturally from organic material under high pressure, such as during the formation of crude oil. Naphthalene is the simplest PAH because its molecule is derived by the fusion of a pair of benzene rings. PAHs range from 'light' substances with average solubility in water to 'heavy' substances with high liposolubility and poor water solubility. PAHs with 3-5 benzene rings are heavy enough to remain in the aquatic system (Farrington and Quinn 1973).

PAHs in the marine environment increase biological oxygen demand (BOD), and are highly toxic to aquatic organisms, and can be carcinogenic to man and animals. They are mutagenic and harmful to reproduction in mammals. Heavy PAHs bind strongly to organic matter in the sea bed thereby contributing to their persistence (Danish EPA, 2003). Heavy PAHs are less soluble in water and are usually present mainly in, or associated with, dispersed oil. Alkylated phenols and aromatic hydrocarbons are the most important contributors to toxicity (Frost et al., 1998). Alkylated phenols have been observed to be responsible for endocrine disruption and potential effects on reproduction. (Frost et al., 1998). However, phenols and alkylated phenols can be degraded by bacteria in sea water and marine sediments (Stephenson 1992).

Trace Metal Content: Trace metals are constituents of produced water and their concentrations in produced water depend largely on the field of operation, particularly with respect to the age and geology of the formation from which oil and gas are produced. Typical metals found in produced water include: lead, manganese, iron, barium and zinc. The metal contaminants in aquatic systems usually remain either in soluble or suspension form and finally tend to settle down to the bottom of the water column or are taken up by marine organisms. The progressive and irreversible accumulation of these metals in the organs of marine organisms ultimately leads to metal-related diseases in the long term because of their toxicity, thereby endangering the aquatic biota and other organisms (Watling 1983; Hart, 1982). Fish may often accumulate large amounts of certain metals

because they are one of the main aquatic organisms in the food chain (Mansour and Sidky 2002; Hadson, 1988). Essentially, fish assimilate these heavy metals through ingestion of suspended particulates or food materials and/or by constant ion-exchange process of dissolved metals across lipophilic membranes such as in gills or adsorption of dissolved metals on tissue and membrane surfaces (Melville and Burchett, 2002). Table 1.2 records the bioaccumulation of trace metals in commercial fish and crabs from the Gulf of Cambay, India in 2006.

Table 1.2 Mean Metal Concentrations (mg/kg dry wt) in Commercial Fishes and Crabs, Gulf of Cambay, India in 2006.

| Heavy Metal | Fish | Crab |
|-------------|-------------------|--------------------|
| As | 1.74 ± 0.856 | ND |
| Cd | 0.23 ± 0.029 | 1.600 ± 0.566 |
| Co | 0.24 ± 0.057 | 0.425 ± 0.177 |
| Cr | 0.77 ± 0.054 | 2.075 ± 0.389 |
| Cu | 2.37 ± 0.451 | 175.45 ± 2.45 |
| Fe | 94.35 ± 2.64 | 155.85 ± 5.10 |
| Hg | 0.97 ± 0.332 | 4.000 ± 0.023 |
| Mn | 12.14 ± 0.72 | 12.270 ± 0.884 |
| Ni | ND | 3.150 ± 0.041 |
| Pb | 1.09 ± 0.071 | 2.775 ± 0.177 |
| Zn | 38.24 ± 1.641 | 44.22 ± 1.21 |

Source: (Mallampati et al., 2007).

Concentration of all Metals is in mg/kg. ND, (Not Detectable).

Produced Solids: In the course of production and well bore operations, produced water can contain precipitated solids, sand and silt, carbonates, clays, bacteria, waxes, asphaltenes, corrosion products and other suspended solids. Produced solids can cause the well or produced water treatment system to shut down. Fine-grained solids can reduce the removal

efficiency of oil/water separators, leading to excesses in oil and grease in discharged produced water (Cline 1998). Solids can form oil sludge in production equipment and will require periodic removal and disposal. The total suspended solids content of discharges in the North Sea varies from about 3 to 85mg/l (E & P Forum, 1994).

Naturally Occurring Radioactive Material (NORM): Naturally Occurring Radioactive Material (NORM) originates from geological formations and can be brought to the surface with produced water. The most abundant NORM compounds present in produced water are radium 226 and radium 228, derived from radioactive decay of uranium and thorium associated with certain rocks and clays in the hydrocarbon reservoir (Utvik 2003). Soluble radionuclides in produced water have not been studied as well as the insoluble low-specific activity scale that precipitates from produced water. According to Michalik (2008), three early studies on soluble radionuclides in produced water have been reported. These studies showed activity concentrations of soluble radionuclides ranging from 0.1-1620 pCi/L for ^{226}Ra and from 8.3-1507 pCi/L for ^{228}Ra . The main factors determining related environmental risk of NORM are the scale of occurrence and level of materials collected at one site (Michalik, 2008).

Residual Production Chemicals: Production chemicals are added at various stages in the production of crude oil and residues may subsequently be present in produced water, which may significantly affect its environmental impact when discharged. Chemicals are used to accomplish several functions such as breaking emulsions to aid oil and water separation, preventing the formation of water-formed scales, controlling the growth of bacteria in producing wells and production systems and aiding in the treatment of water to remove oil. Biocides, emulsion breakers (normal and reverse), scale and corrosion inhibitors, coagulants, flocculants, antifoam, surfactants and paraffin control chemicals are examples of treatment chemicals. Addition of more than one chemical to a system is a common practice amongst operators in oil and gas exploration and production. Virtually all production chemicals are complex formulations of compounds manufactured from impure raw materials. The formulations sold by the chemical supply companies usually contain several compounds as constituents. Besides the 'active' ingredient(s), normally there will

be a solvent. Manufacturers may also include other compounds to augment a specific action of the main ingredient, small amounts of compounds with different functions, other solvents, or other chemicals added to allow better achievement of the primary purpose. The generic chemical type is defined by the functional group(s) of the main active ingredient(s). The complex nature of these formulations can make rigorous determination of solubility, composition, distribution coefficient, etc., virtually impossible in a classical sense (Hudgins, 1994; Henderson, 1999). Some or all of these chemicals may be discharged to the marine environment along with the produced water (Stephenson, 1992; Slager, 1992; and Flynn et al., 1996). In the North Sea an estimated 5934 tonnes of production chemicals were discharged in 1989 along with an estimated 84,097 tonnes of drilling chemicals (Hudgins, 1994).

1.2.3 Types of Production Chemicals

There exist a wide range of chemicals used in oil production to perform various preventive and control functions.

Scale Inhibitors: A thermodynamic chemical equilibrium is often created between dissolved solids in produced water and downhole conditions. As produced water is generated and it reaches the surface, its temperature and pressure become lowered thereby altering the chemical equilibrium that was present earlier. This results in the precipitation of inorganic salts in the production equipment, rendering it unfit for use. Scale deposition inside flow lines and equipment occurs most often when the formation water becomes supersaturated with the carbonate or sulfate salts of barium, calcium and strontium. This may occur due to changes in temperature and pressure of the fluids in well tubulars, flow lines and process equipment, or when non-compatible water is mixed. The latter scenario can be caused by injected sea water breaking through to the producing well and mixing with barium or strontium-rich formation water (Callaghan, 1991; Henderson, 1999). A minimum concentration of scale inhibitors, typically 3 to 10ppm, must be present at all times to prevent scale deposition. Scale inhibitors added to produce water-treating equipment will be discharged primarily with the water. Polyphosphate esters of amino-

alcohols, phosphonates or acrylic acid type polymers (sodium polyacrylate polymers) are examples of organic chemicals used in scale inhibition. These chemicals absorb into the crystal nuclei as scale first precipitates and prevent further deposition (Jones, 1988).

Biocides: The growth of bacteria in produced water can produce hydrogen sulphide by biochemical reduction of sulphates. Dissolved H₂S makes produced gas highly corrosive. Also the presence of bacteria can impact production operations, leading to the occurrence of bacterial fouling of equipment and hydrocarbon degradation. In addition, clusters of iron sulphide and degraded oil can be formed at the oil/water interface in tanks and separators reducing their efficiency and effectiveness. Various bacteria, especially sulfate-reducing bacteria, must be controlled to prevent corrosion or fouling of the equipment or wells. Aldehydes, quaternary ammonium salts, and amine acetate salts are the most commonly used biocides. Intermittent slug treatments at 50 to 200 ppm of formulation are used to obtain good control with minimum total biocide usage. All the biocides are highly water soluble, as they are designed to be effective in the water phase (Reis, 1996).

De-oilers or Reverse Emulsion Breakers: Produced water often contains an oil-in-water emulsion. Water that is separated from oil often contains finely dispersed oil droplets, which have been carried over as a reverse (oil-in-water) emulsion. It is normally necessary to reduce the residual amount of oil prior to sea disposal or re-injection. Re-injection of water containing dispersed oil together with suspended solids often leads to plugging of the formation and consequently loss of injectivity (Callagan, 1991). For discharge to the North Sea in the United Kingdom, the dispersed oil content is subject to a maximum of 30mg/l by legislation. Reverse breakers are used to remove oil droplets from produced water.

The first stage of the process of de-oiling is normally gravity separation, using plate separators offshore. In order to lower the electrostatic forces on the oil droplets and to allow them to coalesce into larger droplets, polyamine or polyamine quaternary ammonium compounds may be added. Low molecular weight (2000-5000) polyamines and polyamine quaternary ammonium compounds are the two most common generic types of de-oilers. Both types are highly water soluble. Other generic types of de-oiling compounds are alkyl quaternary ammonium salts, alkyl benzyl ammonium quaternary ammonium salts and

acrylic sulphonates. Some formulations include moderately high concentrations of aluminum, iron or zinc chlorides. Dosages of 5 to 25ppm may be required, with perhaps half distributing into the discharged water. (Callagan, 1991).

Antifoams: During production operations, crude oils generate foam. The foam generated shows inhibitory effects to the separation of oil, water and solids in production equipment. Foam breakers such as silicones, polyglycol esters and aluminum stearate are often added to topside equipment to mitigate foam formation. In an oil separator, both types will tend to distribute into oil, but their solubility is rather low (Jones, 1988).

Surfactants: While water-soluble surfactants may be a component in many formulations, they are rarely used as a primary treating chemical. Detergents, however, are used offshore for cleaning equipment and decks to meet regulatory requirements. The two most common types are the alkyl aryl sulphonates and the ethoxylated alkylphenols, both of which are widely used in other industrial and household applications. Oil-soluble versions are available for maintenance of tank and vessel interiors (Henderson, 1999).

Demulsifiers: Oil is normally produced together with dissolved or free gas and water. Traditionally, the gas is separated first at one or two pressure levels, followed by the separation of water at approximately atmospheric pressure. When a large proportion of water is present, some will be in the form of 'free' water which separates easily from the oil. Some water will be present as an emulsion in the oil, which is stabilised by naturally – occurring surface active compounds in the crude oil or added chemicals such as corrosion inhibitor, and finely divided solids, which adsorb at the oil-water interface. Different essential steps are needed to dehydrate crude oil, including destabilisation of the emulsion, coalescence of the destabilised water droplets and separation of the water from the oil phase. The vast majority of operators use a demulsifier to destabilise water/oil emulsions (Hudgins, 1994). Demulsifiers are blends of surface active agents, normally supplied in a solvent. Anionic, cationic and non-ionic demulsifiers can be used, depending on the specific nature of the emulsion. The most common compounds are oxyalkylated alkyl phenol formaldehyde resins, polyglycol esters, and alkyl and aryl sulphonates. Almost all

formulations contain more than one of these generic types as well as surfactants. The components of these formulations tend to be very insoluble in water and distribute into the oil phase. Typical use concentrations are about 25 to 100 ppm based on oil, with an estimated 0.4 to 4ppm distributing into produced water.

Coagulants and Flocculants: Low oil levels are often achieved with the use of a flotation unit, which may require the addition of a polyelectrolyte flocculant to maintain a stable froth that can be skimmed off. Other de-oiling techniques such as coalescers and filters may also use coagulants and flocculants to improve the oil-water separation and remove suspended solids from water before injection into the reservoir. Coagulants and flocculants tend to be generically similar to polyamine quaternary ammonium de-oilers, although inorganic salts occasionally may be used in water treatment. Flocculant molecular weights typically range from 0.5 to 20 million, with the coagulant molecular weights more comparable to those of de-oilers. Both coagulants and flocculants are water soluble. Typically, 5 to 10 ppm is used to remove oil from water. Much lower concentrations may be required (e.g., 0.1 to 1ppm) when filtering suspended solids from injection water (Hudgins, 1994).

Paraffin Treating Chemicals (Wax Inhibitors, Wax Solvents and Pour Point Depressants): In many crude oils, crystallisation of aliphatic long chain hydrocarbons (wax) occurs as the temperature decreases from the reservoir to the stock tank. In addition, reduction in flow and eventually plugging of pipelines and facilities can occur. Accumulated solid hydrocarbons in the production system are controlled by the use of paraffin-treating chemicals. These types of chemicals restrict the growth of paraffin crystals by reducing the tendency of the crude oil to deposit. The most commonly used compounds include vinyl polymers, sulphonate salts and mixtures of alkyl polyethers and aryl polyethers. Paraffin solvents are used to remove accumulations of deposits. The solvents usually are refinery cuts and may be primarily aliphatic or aromatic depending on the nature of the deposits. All these class of chemicals are far more soluble in oil than in produced water (Henderson, 1999).

Corrosion Inhibitors: Corrosion is one of the most serious problems in offshore operations. Production facilities are subject to corrosive attack by carbon dioxide, hydrogen sulfide and other aggressive constituents that may be present in the reservoir fluids. Coatings, cathodic protection and materials selection are used in the control of external corrosion, with corrosion inhibitors being used to supplement other methods for internal corrosion control. A corrosion inhibitor is a chemical compound that, when added in small concentration stops or slows down corrosion which is defined as deterioration of intrinsic properties in a material due to reactions with its environment (Jones, 1988). A typical good corrosion inhibitor will give 95% inhibition at a concentration of 80 ppm, and 90% at 40 ppm (Jones, 1988). Some of the mechanisms of inhibitory effects are formation of a passivation layer (a thin film on the surface of the material that stops access of the corrosive substance to the metal), inhibition of either the oxidation or reduction part of the redox corrosion system (anodic and cathodic inhibitors), or scavenging of the dissolved oxygen. Corrosion inhibitors can either be oil-soluble/water dispersible or water-soluble/oil dispersible. The choice of type depends upon whether the metal or alloy to be protected is water-wet or oil-wet, although in general oil-soluble inhibitors are preferred for oil production because of their greater effectiveness. All the corrosion inhibitors used offshore are organic compounds that form a protective layer on the target metal surface. Most contain nitrogen as the key functional group. This nitrogen-containing material reacts with a carboxylic acid to form a compound that can be optimized for a variety of different applications by altering the reaction conditions (Henderson, 1999). Corrosion inhibitors can commonly be classified by the following generic types, based on the chemical structure of their active component(s):

Amide/imidazoline: These are complex mixtures and most corrosion inhibitor formulations have one or both compounds present. These compounds usually have high molecular weight and low solubility in water.

Amine/amine Salts: This salt mixture contains mostly long chain monoamines (C10-C18) and solubility in water depends on the length of and degree of ionisation of the carbon tail. Ethoxylation of the active sites increases water solubility regardless of the pH.

Nitrogen Heterocyclics: In these compounds, nitrogen may be incorporated into an aromatic or aliphatic ring structure and they usually have little or no water solubility.

Quaternary Ammonium Salts: Quaternary ammonium-based surfactants mainly produced from natural fats and oils, resulting in mixed alkyl chain lengths in some products (GarcõÂa et al., 2001). Quaternary ammonium compounds (cationic surfactants) have been used for more than 30 years in large quantities (Garcia et al, 2000 and 2001; Callely and Foster, 1977; Kaech and Egli, 2001; Valle's et al., 2000, and van Ginkel, 1995). The active component in quaternary ammonium compounds (QACs) may be a molecule like trimethylalkylammonium chloride, with an alkyl side chain most likely comprising a complex mixture of long chain hydrocarbons. All quaternary ammonium salts have high water solubility (Vik and Bakke, 1992; Henderson, 1999). QACs contain four functional groups attached covalently to a positively charged central nitrogen atom (R_4N^+). These functional groups (R) include at least one long chain alkyl group and the rest are either methyl or benzyl groups. An example of a quaternary ammonium salt is benzyl dimethylhexadecylammonium chloride [BDHAC] represented by the structural formula (Figure 1.2) and 3D molecular structure (Figure 1.3).

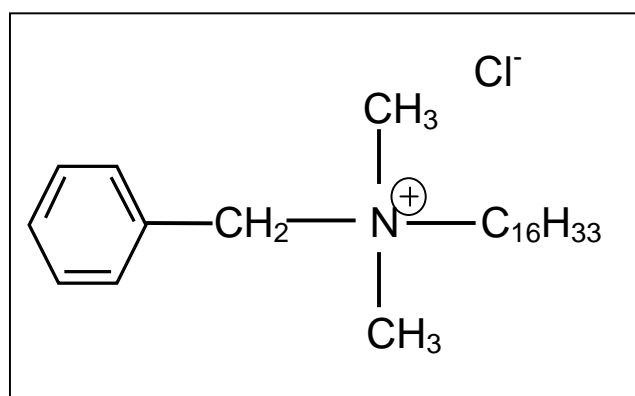


Figure 1.2 Structure of Benzyl Dimethylhexadecylammonium Chloride [BDHAC]

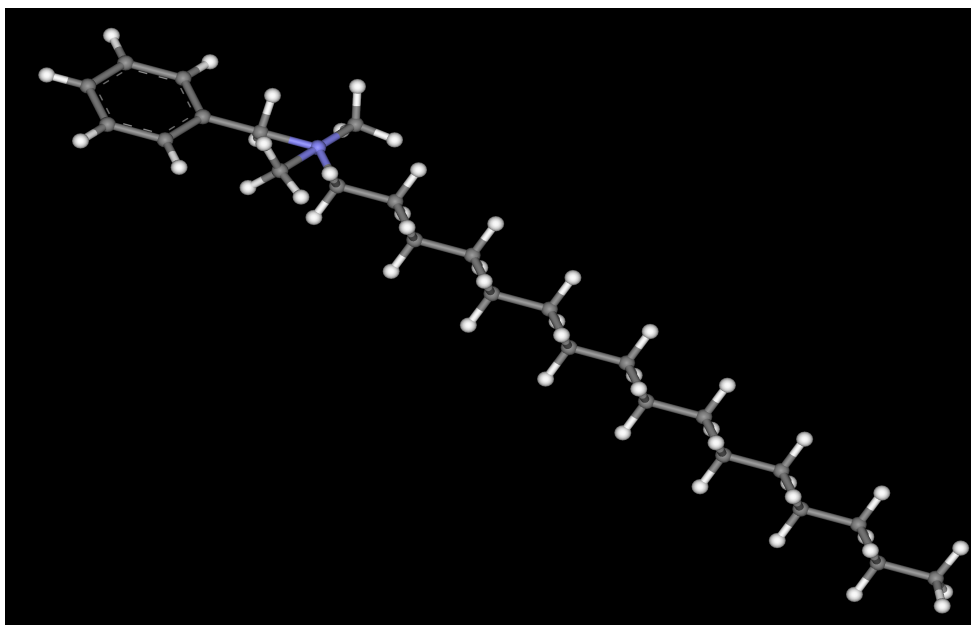


Figure 1.3 3D Structure of Benzyl Dimethylhexadecylammonium Chloride [BDHAC]

Quaternary ammonium compounds (QACs) containing a long-chain alkyl group and/or a benzyl group, are cationic surfactants and the active ingredient in many corrosion inhibitor formulations. QACs are also used in domestic and industrial applications as surfactants, emulsifiers, fabric softeners and disinfectants (Garcia et al., 1997; Patrauchan and Oriel, 2003).

QACs combine disinfecting, wetting, frothing, anti-corrosion and waterproofing properties and are extensively used in biochemical and pharmaceutical applications (Parr et.al., 1998; Verbina, 1973). Because of their widespread application and volume as cationic surfactants, QACs are released and accumulated in aquatic environments, where toxicity to aquatic organisms can occur

(Beveridge et al. 1998; Boething 1984; Tubbing and Admiraal 1991). Environmental effects of such release are important because not only are QACs toxic to micro-organisms and animals, they also exert secondary effects. The biocidal action of QACs can lead to lowered degrading activities of micro-organisms, not only on QACs themselves but also toward other chemicals (Nishihara et al. 1998).

1.3 Bacteria in the Environment

Bacteria are single-celled organisms found in most environments. They are a large group of unicellular, prokaryote, microorganisms which are typically a few micrometers in length. They have a range of shapes, such as spheres, rods and spirals. Bacteria are ubiquitous in different habitats on earth (Fredrickson et al., 2004). Bacteria present in the environment are classified based on shapes, Gram stain, oxygen requirement, growth and reproduction and phyla (Fredrickson et al., 2004).

Based on shape, bacteria can be broadly divided into the following groups: rod-shaped bacteria (*Bacilli*), sphere-shaped bacteria (*Cocci*) and spiral-shaped bacteria (*Spirilla*). They can also be classified on the basis of Gram stain in which an agent is used to bind to the cell wall of the bacteria. They can be Gram-positive or Gram-negative (Jenkins, et al., 1993). Bacteria can be classified based on requirement of oxygen for survival i.e. aerobic bacteria (requires oxygen) and anaerobic bacteria (does not require oxygen). Classification based on growth and reproduction groups bacteria into autotrophic (obtain carbon and/or sugar from sunlight or chemical reactions) and heterotrophic (obtain carbon and/or sugar from the environment) while that based on the *Phyla* is determined through morphology, DNA sequencing, and biochemistry (Jenkins, et al., 1993). They include *Aquificae*, *Bacteroids*, *Chlorobia*, *Chrysogenetic*, *Cyanobacteria*, *Fibrobacter*, *Firmicutes*, *Flavobacteria*, *Fusobacteria*, *Planctomycetes*, *Proteobacteria*, *Spirochaetes* and *Thermomicrobia*. Each phylum can be related to a number of species and genera of bacteria. These classifications basically include those bacteria which are found in different environments, such as ocean, sea, highly acidic environment, and highly alkaline environment (Fredrickson et al., 2004).

In order to grow, a bacterium must have an energy source, a source of carbon and other required nutrients, and a range of physical conditions such as O₂ concentration, temperature, and pH. Bacteria are sometimes referred to as individuals or groups based on their growth patterns under different chemical (nutritional) or physical conditions. For example, phototrophs are those organisms that use light as an energy source while thermophiles are those organisms that grow at high temperatures. Organisms that use radiant energy (light) are called phototrophs while organisms that oxidise an organic form of carbon are called heterotrophs or (chemo) heterotrophs. Organisms that oxidize inorganic compounds are called lithotrophs (Leahy and Colwell, 1990).

The carbon requirements of microorganisms must be met by organic carbon or by CO₂. Microorganisms that utilise CO₂ as a sole source of carbon for growth are called autotrophs. There are self-nourishing, obligate and facultative autotrophs. Self-nourishing autotrophs are those organisms that are able to use CO₂ as a sole source of cell carbon; obligate autotrophs do not have appropriate transport system and so cannot utilise organic compounds, while facultative autotrophs are able to use other sources of organic substrates (Annweiler et al., 2000).

Bacteria present in sediment may be limited in the amount of utilisable carbon present with respect to other nutrients such as nitrogen, phosphorus, sulphur and other trace metals needed for the production cell structures and energy. Thus, if a new source of carbon is introduced in the form of an organic pollutant, cells capable of extracting carbon from such compounds will survive (Annweiler et al., 2000; Prenafeta-Boldu et al., 2001). Such cells capable of metabolising the organic pollutant can increase from near non-detectable levels to dominance. (Leahy and Colwell, 1990). Bacteria respond to stress such as pollutants by attempting to correct the pollutant perturbation in the environment by increasing the probability of survival (Schlegel, 1985). Some bacteria are protected by their outer membrane, which prevents the penetration of many types of chemicals. (Heinzel, 1988; Russell, 1992 a, b).

The Bacterial cell

The fundamental divisions of the bacterial cell are the cell wall, cytoplasmic membrane and cytoplasm. The structure and composition of the cytoplasm as well as the cytoplasmic

membrane are conserved between different types of bacteria. The bacterial outer envelope is responsible for the responses to biocidal challenges. For example, the outer membrane of Gram-negative bacteria acts as permeability barrier and is responsible for the resistance of these micro-organisms to antimicrobial compounds (Gilbert et. al. 1990). The cytoplasmic membrane is made up of phospholipid bilayer with embedded proteins. It regulates the transfer of solutes and metabolites in and out of the cell cytoplasm. Bacterial cytoplasm contains the cell nucleic acid, ribosomes and different enzymes. The bacterial cell wall lies outside the cytoplasmic membrane and provides strength and shape to the cell. On the basis of simple staining procedure, cells of bacteria can be divided into two major groups: Gram positive and Gram negative. The staining procedure reveals differences in wall structure and organisation between these groups. A structural polymer called peptidoglycan gives strength to the cell walls of Gram positive and Gram negative bacteria (Wilson et al. 2002).

Gram-positive Cell Wall

The main component of the Gram-positive bacterial cell wall is the peptidoglycan. Other components include teichoic acid and lipoteichoic acid (Figure 1.4).

Gram-negative Cell Wall

The cell wall of the Gram-negative bacteria has a more complex multilayered structure than that of Gram-positive species (Figure 1.5). The cell wall has a much thinner peptidoglycan layer. The peptidoglycan is hydrated, with low degree of cross-linking and is attached to the outer membrane by lipoprotein (Wilson et al. 2002). The periplasm is the region between the cytoplasmic and the outer membranes. The outer leaflet of the cell wall is composed of lipopolysaccharide (LPS).

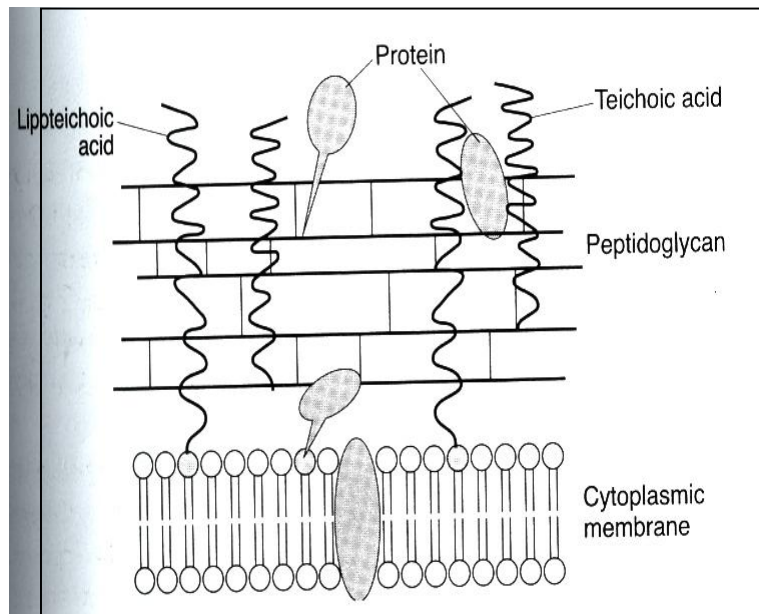


Figure 1.4 Cross-section through the wall of a typical Gram-positive bacterium. Peptidoglycan is the main constituent of the wall and is thicker than in the case of Gram-negative species. Source: (Wilson et al. 2002).

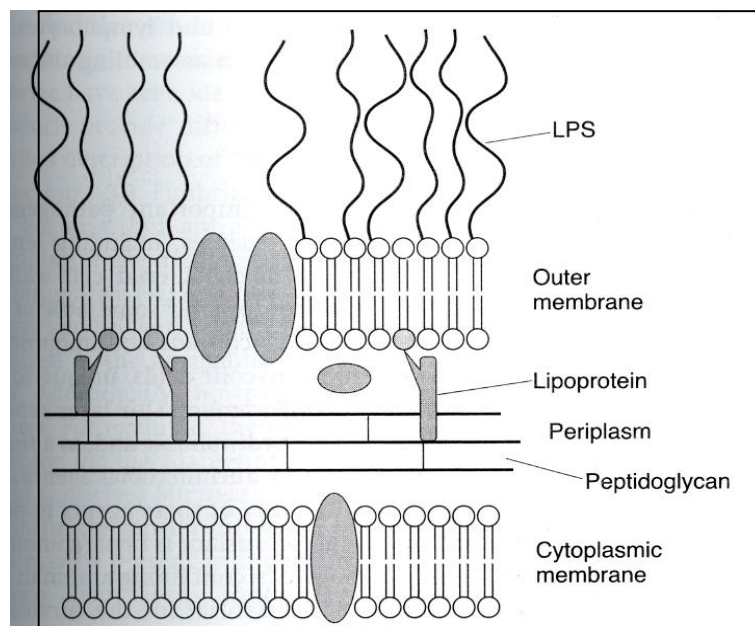


Figure 1.5 Cross-section through the wall of a typical Gram-negative bacterium. The wall is multilayered and consist a thinner layer of peptidoglycan than in the case of Gram-positive species. Source: (Wilson et al. 2002).

Bacteria in Activated Sludge: The activated sludge process is a method for the treatment of wastewater. It is a process in which the carbonaceous organic matter of wastewater provides an energy source for the production of new cells for a mixed population of microorganisms in an aquatic aerobic environment. The microorganisms convert carbon into cell tissue and oxidised by products that include carbon dioxide and water. Bacteria constitute the majority of microbes present in activated sludge (Chipasa and Medrzycka 2004a, b; Nielsen and Nielsen 2002; Nielsen et al. 2004; Wagner and Loy 2002). The composition and activity of the microbial community in activated sludge is essential for waste treatment, therefore the analysis of micro-organisms present is very important (Nielsen et al. 2004). Many different bacterial groups are present as either single cell microcolonies or as filamentous bacteria (Nielsen et al. 2004). Majority of the bacteria in activated sludge are aerobic heterotrophs, (Nielsen et al. 2004; Wagner and Loy 2002).

A large number of bacteria have been isolated from activated sludge and the genera includes *Pseudomonas*, *Bacillus*, *Achromobacter*, *Enterococcus*, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Arthrobacter*, *Escherichia*, *Salmonella*, *Proteus*, *Streptococcus*, *Staphylococcus*, *Micrococcus*, *Corynebacterium*, *Clostridium*, *Penicillium* (Mehandjiyska, 1995). Researchers have also reported that the majority of bacteria in activated sludge are Gram-negative bacteria (Foster, 1977; Sharifi-Yazdi et al. 2001). Bacteria present in activated sludge are responsible for the degradation of organic and inorganic compounds. They derive nutrients from the compounds available in the waste. They are able to synthesise their enzymes, metabolic intermediates, structural proteins, lipids and nucleic acids from carbon compounds. They oxidise either organic compounds (chemoorganotrophic metabolism) or inorganic compounds (chemolithotrophic metabolism) to obtain energy and use the energy for reproduction and growth (Sharifi-Yazdi et al. 2001).

Activated sludge consists of a mixed population of microorganisms, and has been reported in many studies to be a source of heterotrophic bacteria for QAC degradation. Several researchers have reported the isolation of bacteria capable of degrading QAC from activated sludge. For example, Nishihara et al., (2000) in the Biodegradation of didecyldimethylammonium chloride isolated *Pseudomonas fluorescens* TN4 from activated sludge, and in another study on the biodegradation of dodecyltrimethylammonium bromide,

Nishiyama and Nishihara (2002) also isolated *Pseudomonas fluorescens* F7 and F2 from activated sludge. However, for BDHAC degradation studies, bacteria were isolated from marine sediment.

1.4 Bacteriocidal Properties of Compounds

Bacteriocidal compounds or biocides may be broadly classified as antiseptics and disinfectants. They are used to kill or restrict the growth of micro-organisms when applied to a living tissue or inanimate objects. An antiseptic is a chemical compound inhibits the growth of micro-organisms when applied to a living tissue while a disinfectant prevents infection by the destruction of harmful micro-organisms when applied to inert objects (Husain, 2008).

The following sequence of events occur when microorganisms are exposed to bacteriocidal compounds such as QACs: adsorption and penetration of the agent into the cell wall, reaction with the cytoplasmic membrane (lipid or protein) followed by membrane disorganisation, leakage of intracellular low-molecular-weight material, degradation of proteins and nucleic acids and lysis of cell wall caused by autolytic enzymes, (autolytic enzymes are enzymes that digest the cell in which they are produced, usually marking the death of the cell) these result in loss of structural organisation and integrity of the cytoplasmic membrane in bacteria, together with other damaging effects to the bacterial cell (Husain, 2008). QACs have bacteriocidal properties and their structural functionality, especially the role of chain length on activity against different bacteria, has been observed (Tomlinson et al., 1977). They are believed to be metabolically specific in their antibacterial action. Their bacteriocidal effect has been attributed to a general dissolution of the cell membrane followed by an inactivation of cytoplasmic enzymes as concentrations increase (Hugo, 1967; Koike and Matsuo, 1969).

Hamilton, (1968) in a study on the mechanism of the bacteriostatic action of tetrachlorosalicylanilide, reported that a common feature of QACs is their ability to cause cell leakage and membrane damage, due to their adsorption to the bacterial membrane in large amounts. Also, Ahlström et al., (1999) stated that QACs with a C₁₆ hydrophobic tail length affected the outer membrane of gram-negative bacteria more extensively than

shorter-chain compounds, possibly due to the strong interaction of the C₁₆ chain with the fatty acid portion of lipid. These authors also mentioned that monoalkyl QACs bind by ionic and hydrophobic interactions to microbial membrane surfaces, with the cationic hydrophilic head group facing outwards and the hydrophobic tails inserted into the lipid bilayer, causing the rearrangement of the membrane and the subsequent leakage of intracellular constituents. QACs are membrane-active agents with a target site at the cytoplasmic (inner) membrane in bacteria (White and McDermott 2001; Russell, 2002).

1.5 Microbial Degradation of Quaternary Ammonium Compounds

In today's more environmentally conscious world, production chemicals are increasingly being scrutinized for possible adverse toxicological and environmental effects. Concerns can arise when production chemicals have the potential to bioaccumulate, or cause endocrine disruption. Endocrine disruption is a process whereby exogenous substances that act like hormones in the endocrine system of organisms, disrupt the physiologic function of hormones. (Diamanti-Kandarakis, et. al., 2009). QACs, which are widely used synthetic organic chemicals, are ingredients in a variety of household products and knowledge of the ultimate biodegradability of these chemicals is important in order to assess the self cleaning function of nature (van Ginkel et.al. 1992). The complete metabolic steps necessary for QAC mineralisation by pure cultures is not known and the fate on release into the marine environment of QACs is poorly understood (Takenaka, 2007). In particular, their rate of degradation, and the nature of any resulting metabolites, remains nebulous. Researchers are interested in studying quaternary ammonium compounds (QACs) for various reasons. Their cationic nature together with their hydrophilic and hydrophobic properties renders them ideal for use as surface active agents and surface modifiers. Studies of quaternary ammonium salts released into the environment have suggested they are degraded and removed in the biological process of a sewage treatment plant (Fenger, 1973; Gerike, et al. 1978; Topping and Water 1982; Games et al. 1982 and Sullivan, 1983).

Biodegradation can be defined as the biologically catalysed reduction in complexity of chemicals. Micro-organisms are frequently the sole means of converting synthetic chemicals into organic products. It is because of their ability to mineralize anthropogenic

compounds that microorganisms play large roles in soil, water and sediments. Many synthetic molecules discharged into these environments are directly toxic or become hazardous following biomagnification. Biomagnification, also known as bioamplification, is the increase in concentration of a substance that occurs in a food chain as a consequence of persistence (slow to be broken down by environmental processes). Mineralization by micro-organisms results in total destruction of the parent compound. Toxicological studies have brought forward approaches that have revealed that chemicals previously deemed to be safe are in fact harmful, thus mineralization is very important in ridding the environments of possible hazards to humans, animals and plants.

Microorganisms carry out biodegradation in many different types of environment. Of relevance to pollutants are sewage treatment systems, soils, underground sites for the disposal of chemical wastes, groundwater, surface waters, oceans, sediments and estuaries. A variety of pollutants are retained by the sediments below marine waters, and these sediments contain large and metabolically active communities of heterotrophic microorganisms. Heterotrophic microorganisms are those organisms that utilise organic matter synthesized by other organisms for energy and growth. Natural communities of micro-organisms in various habitats have an amazing physiological versatility. They are able to metabolize and often mineralize a range of organic molecules. Probably every natural product regardless of its complexity is degraded by one or another species in some particular environment (Alexander 1973; Hanson 1997). Microbial degradation of chemicals in the environment is an important route for removal of these compounds. The biodegradation of these compounds is often a complex series of biochemical reactions and is often different when different microorganisms are involved.

Biodegradation of quaternary ammonium compounds by microorganisms has been identified in the Organisation for Economic Co-operation and Development/ European Economic Community (OECD/EEC) screening test (Larson, 1983, Masuda et.al, 1976). However, the results from these simple biodegradation tests revealed the formation of recalcitrant intermediates. Description of the degradation pathways of organic compounds is a vital proof of their total mineralization. Figure 1.6 illustrates the possible mechanisms of QACs degradation by bacteria. The mechanisms include ω - oxidation, β oxidation, C-N fission and ring cleavage.

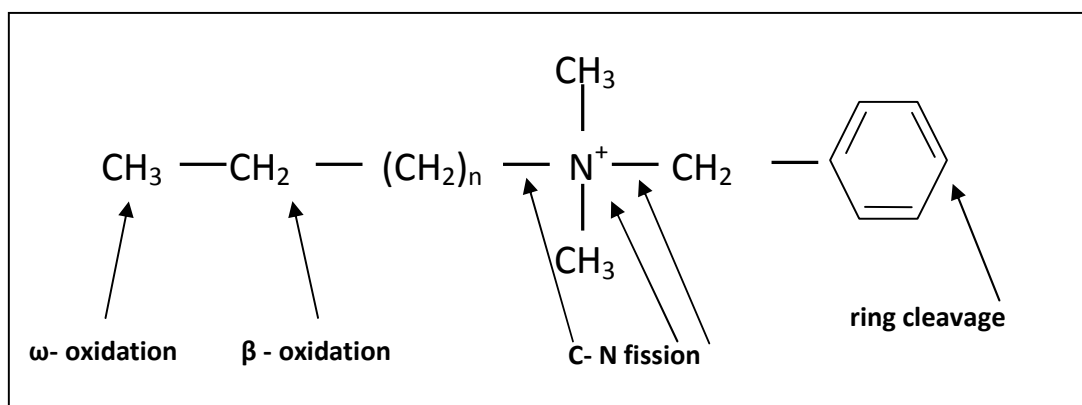


Figure 1.6 Possible Mechanisms of Degradation of QACs by Bacteria

Only few reports are available regarding biodegradation of QACs. Gawel and Huddleson (1972) observed that tetradecylbenzyltrimethylammonium chloride at a concentration of 10mg/l was biodegradable in a shake flask culture. The authors did not state the source of the culture. Confirmation of QAC disappearance in the flask was based on colorimetric and UV spectrometric analysis. Substitution of the ethyl group attached to the benzyl ring delayed the rate of biodegradation, although 95% biodegradation was recorded in 48 hours of incubation. Also, Hampton and Zatman (1973) studied the metabolism of tetramethylammonium chloride by bacterium 5H2 isolated from soil. The organism was gram-negative, non-motile and rod-shaped and was capable of growing on tetramethylammonium chloride and trimethylamine when they were present as the sole source of carbon. They observed that the tetramethylammonium chloride was completely biodegraded to methylamine via the intermediates of trimethylamine and trimethylamine N-oxide. Fenger et al., (1973) in a continuous flow experiment using preformed activated sludge, observed that the detectable amount of tetradecylbenzyltrimethylammonium chloride was reduced by 85%. Infra-red spectroscopy and gas chromatography were used to detect products of degradation which included debenzylated amine as well as benzoic and acetic acids. In another study on bacterial metabolism of quaternary ammonium compounds, Dean-Raymond and Alexander (1977) reported that a mixture consisting of individual strains of *Pseudomonas* and *Xanthomonas* grew in solutions containing decyltrimethylammonium bromide as sole carbon source. The xanthomonad was able to

utilize the quaternary ammonium compound in the presence of other organic molecules and the intermediates formed from the degradation included 9- carboxynonyl- and 7- carboxyheptyl-trimethylammonium, suggesting the oxidation of the terminal carbon of the decyl functional group and that the resulting carboxylic acid was subject to β -oxidation.

Gerike (1978) investigated the biodegradation of dodecylbenzyltrimethylammonium chloride using an activated sludge developed by OECD. They monitored QAC elimination by observing the formation of disulphine blue active complex. The author compared dissolved organic carbon (DOC) before and after the biological treatment and showed that the test substance was reduced by 54%. Ruiz Cruz (1979) also studied the biodegradation of tridecylammonium chloride in river water at a concentration of 5mg/l. He reported that 50% of the test substance disappeared in 7.4 days. Larson and Vashon (1983), in a study on adsorption and biodegradation of cationic surfactants observed they were ultimately biodegradable when present at low concentrations in river surface water and/or sediments. However, the extent of biodegradation varied depending on the structure and concentration of the QAC, microbial acclimation and presence of QAC resistant/degrading microorganisms. These findings indicated the metabolic fates of QAC in a freshwater environment.

van Ginkel, et al., (1992) investigated the utilization of hexadecyltrimethylammonium chloride by *Pseudomonas* strain B1 as a carbon and energy source. *Pseudomonas* strain B1 was isolated from activated sludge. They observed that the bacterium was able to grow on alkyltrimethylammonium salts (C12 to C22) and possible intermediates of the breakdown of hexadecyltrimethylammonium chloride such as hexadecanoate and acetate were also reported. It was also observed that the bacterium did not grow on amines and adaptation studies carried out suggested that the bacterium was able to oxidize the alkyl chain of hexadecyltrimethylammonium chloride. This was confirmed by the formation of trimethylamine. The study revealed that the initial hexadecyltrimethylammonium chloride oxygenase activity that was measured by the formation of trimethylamine, was NAD(P)H and oxygen dependent and assays of aldehyde dehydrogenase, hexadecanoyl-coenzyme A, dehydrogenase and isocitrate lyase in cell extracts revealed the potential of *Pseudomonas* strain B1 to metabolize the alkyl chain via β -oxidation (Figure 1.7).

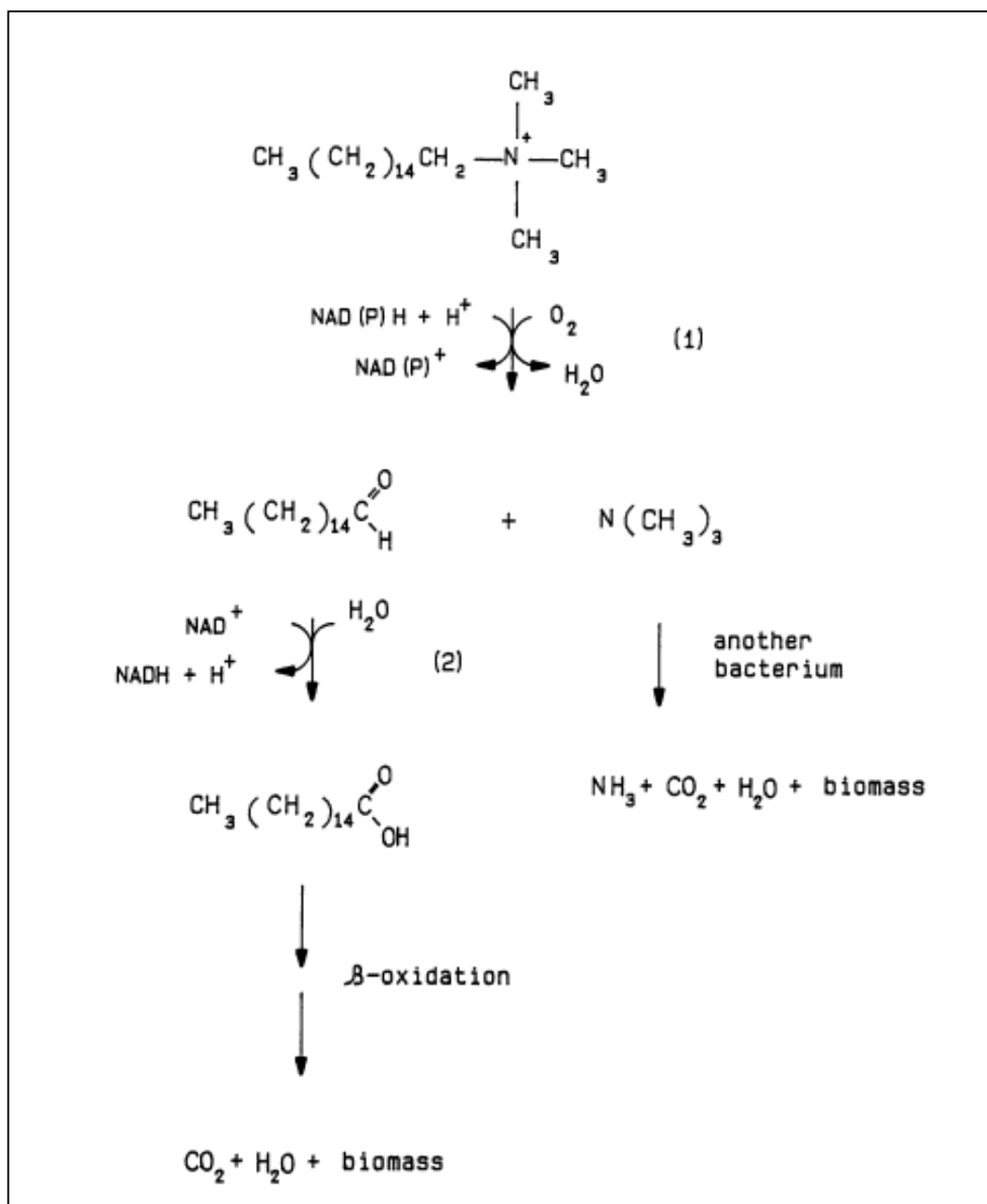


Figure 1.7 Proposed Biodegradation Pathway of Hexadecyltrimethylammonium Chloride by *Pseudomonas* Strain B1: (1) Monooxygenase (2) Alkanal Dehydrogenase (van Ginkel, et al., 1992).

Kroon et al., (1994) studied the metabolism of dodecyldimethylamine by *Pseudomonas* MA3 isolated from activated sludge. The test substance was the only source of carbon and

energy for MA3. Degradation of the alkyl chain by *Pseudomonas* MA3 was as a result of the liberation of dimethylamine by the cleavage of the C alkyl-N bond. Other intermediates that were formed were dodecylamine, dodecanal, decanoic acid and acetic acid. Dodecanal was converted into dodecanoic acid and this intermediate was further degraded via the β -oxidation pathway. Nishihara, et al., (2000) studied the biodegradation of dodecyldimethylammonium chloride (DDAC) by *Pseudomonas fluorescens* TN4 isolated from activated sludge from a municipal sewage treatment plant. DDAC was the sole source of carbon and energy for strain TN4. They observed that *P. fluorescens* TN4 degraded DDAC to produce decyldimethylamine and subsequently, dimethylamine, as the intermediates (Figure 1.8). Also the TN4 strain was able to assimilate other quaternary ammonium compounds i.e. alkyltrimethyl- and alkylbenzyltrimethyl-ammonium salts, but not alkylpyridinium salts. The bacterium was highly resistant to the test substances and utilized them by an N-dealkylation process. Data from their investigation supported the fact that there are some QAC-resistant and QAC-degrading bacteria such as strain TN4 in the environment.

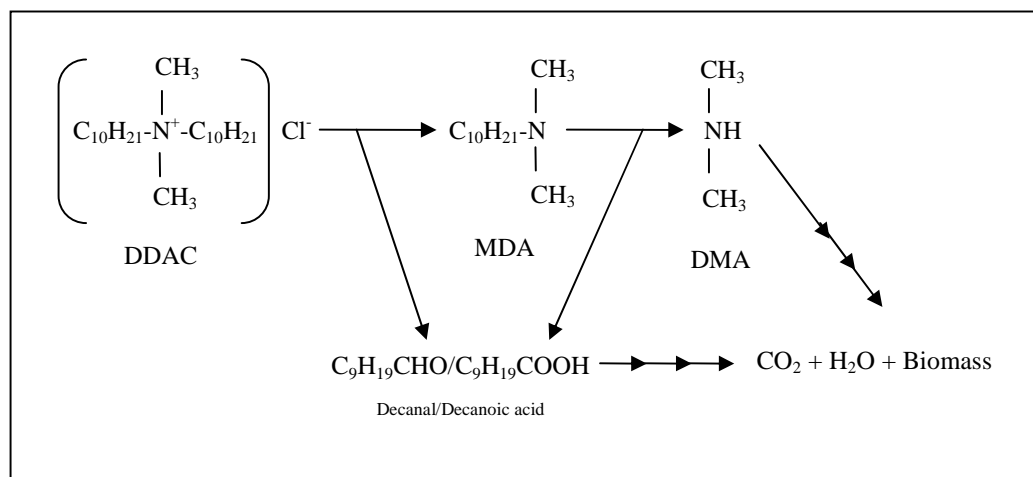


Figure 1.8 Possible Degradation Pathway of DDAC by *P. fluorescens* TN4. (Nishihara, et al., 2000).

In another study on the biodegradation of dodecyltrimethylammonium bromide (DTAB), Nishiyama and Nishihara (2002) isolated two strains, *P. fluorescens* F2 and F7, from activated sludge of a waste water treatment plant (WWTP). They observed that *P. fluorescens* F7 grew in basal medium containing DTAB as the sole carbon source, producing trimethylamine (TMA) from DTAB by N-dealkylation, and the intermediate TMA that was formed was further degraded to dimethylamine (DMA). The report also showed that *P. fluorescens* F2 was able to degrade DTAB to TMA, but that this was not further degraded to another metabolite. The results from their investigation demonstrated that monoalkyl quaternary ammonium compounds were ultimately degraded by N-dealkylation and mineralization by microorganisms isolated from activated sludge.

Patrauchan and Oriel (2003) investigated the degradation of benzyldimethylalkylammonium chloride (BAC) by *Aeromonas hydrophila* sp. K. The organism was isolated from polluted soil and was capable of utilizing BAC as a sole source of carbon and energy. They used high performance liquid chromatography and gas chromatography–mass spectrometry (GC-MS) analysis to study the pathway for BAC degradation by the bacterium. They observed that during BAC biodegradation, there was formation of benzyldimethylamine, benzylmethylamine, benzylamine, benzaldehyde and benzoic acid. It was reported that the formation of benzyldimethylamine as the initial metabolite was as a result of cleavage of the C-alkyl-N bond as the first step of BAC catabolism and liberation of benzylmethylamine and benzylamine likely was as a result of subsequent demethylation reactions, followed by deamination with formation of benzaldehyde (Figure 1.9). They also observed that benzaldehyde was rapidly converted into benzoic acid, which was further degraded. Their findings supported the fact that *A. hydrophila* sp. K was capable of degrading BAC and were also significant for understanding biodegradation pathways of benzyl-containing quaternary ammonium compounds.

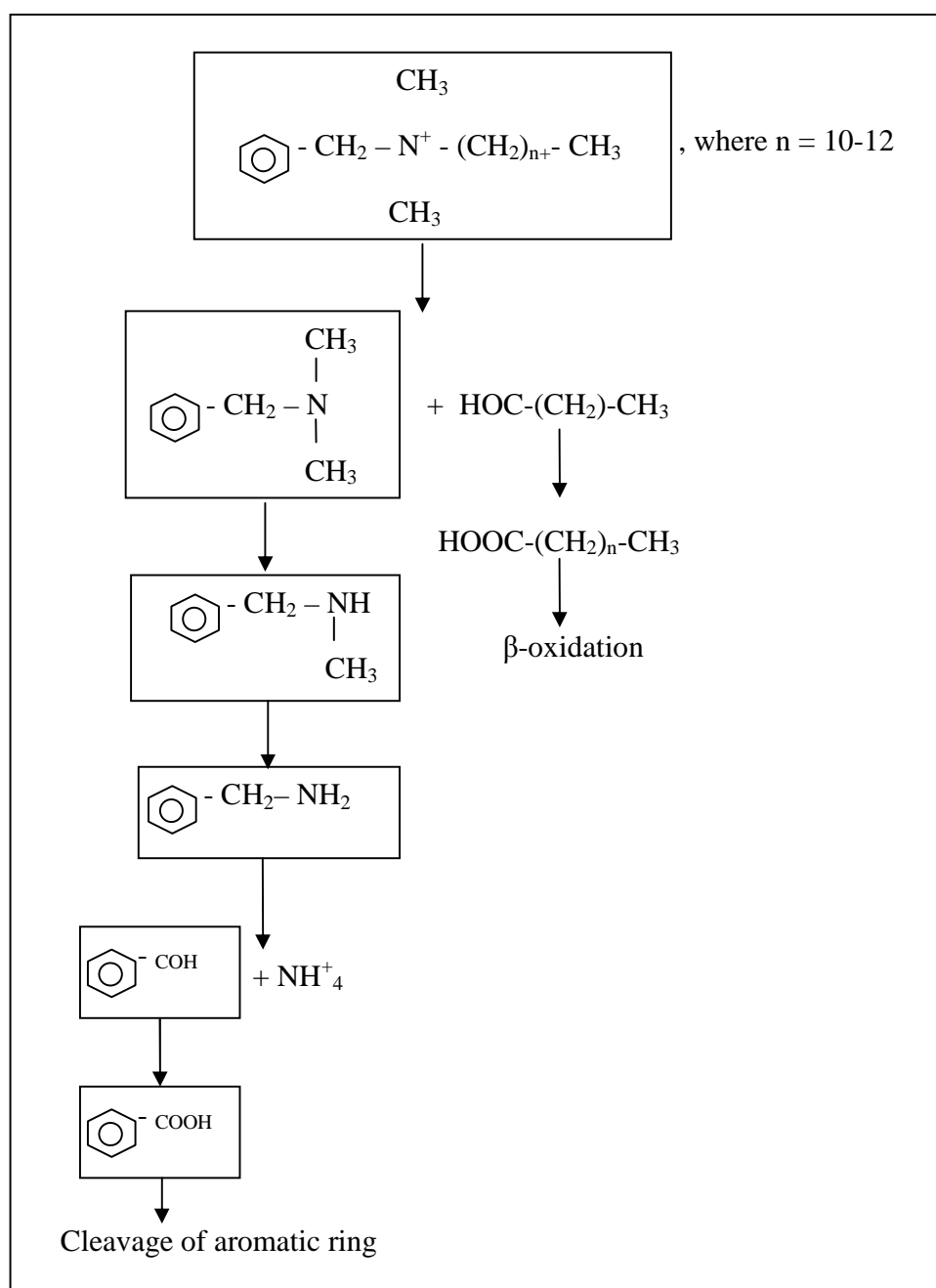


Figure 1.9 Proposed Pathway for Benzyldimethylalkylammonium Chloride Biodegradation by *Aeromonas hydrophila* sp. K (Patrauchan and Oriel, 2003).

Tabata et al., (2003) studied the adaptation mechanism of *Pseudomonas aeruginosa* ATCC 10145 to quaternary ammonium compounds (QACs). They observed that *P. aeruginosa* exhibited remarkable resistance to *N*-dodecylpyridinium iodide (P-12), whose structure is similar to that of a common disinfectant, cetylpyridinium chloride. They also observed an adapted resistance of the strain to benzalkonium chloride (BAC), which is commonly used as a disinfectant. The P-12-resistant strain exhibited cross-resistance to BAC and analysis of the outer membrane protein of the strain by two-dimensional polyacrylamide gel electrophoresis showed a significant increase in the level of expression of a protein (named OprR). They suggested that OprR significantly participated in the adaptation of *P. aeruginosa* to QACs, such as P-12 and BAC.

Also, Yuwono, (2005) isolated an osmotolerant rhizobacterium from a weed rhizosphere which showed tolerance in up to 1.0 M NaCl. The isolate was subjected to growth analysis in a medium which contained 10 mM betaine as the sole carbon source. The author observed that betaine could be used as the sole carbon source for the growth of salt-tolerant rhizobacteria under NaCl-stress at 1.0 M concentration. It was found that betaine at 100 mM concentration suppressed the growth of salt-tolerant rhizobacteria. The growth of the osmotolerant rhizobacterium was stimulated when it was grown in a medium containing both glucose and betaine, suggesting that betaine was an osmoprotectant.

Another investigation by Kaecha, et al., (2005) on the isolation and characterization of heterotrophic bacteria able to grow aerobically with quaternary ammonium alcohols (QAAs), 2,3-dihydroxypropyl-trimethyl-ammonium (TM), dimethyl-diethanolammonium (DM) and methyl-triethanol-ammonium (MM) as sole source of carbon and nitrogen isolated two bacteria with DM, referred to as strains DM 1 and DM 2 respectively. Based on 16S-rDNA analysis, these isolated strains provided 97% (DM 1) and 98% (DM 2) identities to the closest related strain *Zoogloea ramigera*. Using MM, a Gram-negative, non-motile rod referred to as strain MM 1 was isolated. The 16S-rDNA sequence of the isolated bacterium revealed 94% identity (best match) to *Rhodobacter sphaeroides*. It was observed that the isolated strains MM 1 and DM 1 grew exclusively with the QAA which was used for their isolation. DM 2 was able to utilize both DM and TM as sole source of carbon and nitrogen.

In another study on the adaptation of *Escherichia coli* K-12 to Benzalkonium chloride (BC), Bore et al., (2007) described the changes in expression level at the transcriptomic and proteomic level for *Escherichia coli* K-12 as it gradually adapted to tolerance levels of 7–8 times the initial minimum inhibitory concentration (MIC) of the QAC. They concluded that *E. coli* K-12 exhibited high tolerance to BC and acquired several resistance mechanisms.

Takenaka, et al., (2007) were able to isolate *Pseudomonas* sp. strain 7-6, from active sludge obtained from a wastewater facility. They observed that *Pseudomonas* sp. strain 7-6 utilized the quaternary ammonium surfactant, *n*-dodecyltrimethylammonium chloride (DTAC), as its sole carbon, nitrogen, and energy source. They also observed that when the bacterium was initially grown in the presence of 10 mM DTAC, it was unable to degrade DTAC. The strain was cultivated in the presence of gradually increasing concentrations of the surfactant until continuous exposure led to high tolerance and biodegradation of the compound. Gas chromatography-mass spectrometry analysis was used in the identification of five metabolites, and two possible pathways for metabolism of DTAC by the bacterium were proposed (Figure 1.10). In the first pathway, DTAC is converted to lauric acid via *n*-dodecanal with the release of trimethylamine. In the second pathway, DTAC is converted to lauric acid via *n*-dodecyldimethylamine and then *n*-dodecanal with the release of dimethylamine. Among the metabolites that were identified, it was seen that the strain pre-cultivated on DTAC medium was able to utilize *n*-dodecanal and lauric acid as sole carbon sources and trimethylamine and dimethylamine as sole nitrogen sources, but could not efficiently utilize *n*-dodecyldimethylamine. Their findings indicated that the first pathway was the main pathway for the degradation of DTAC.

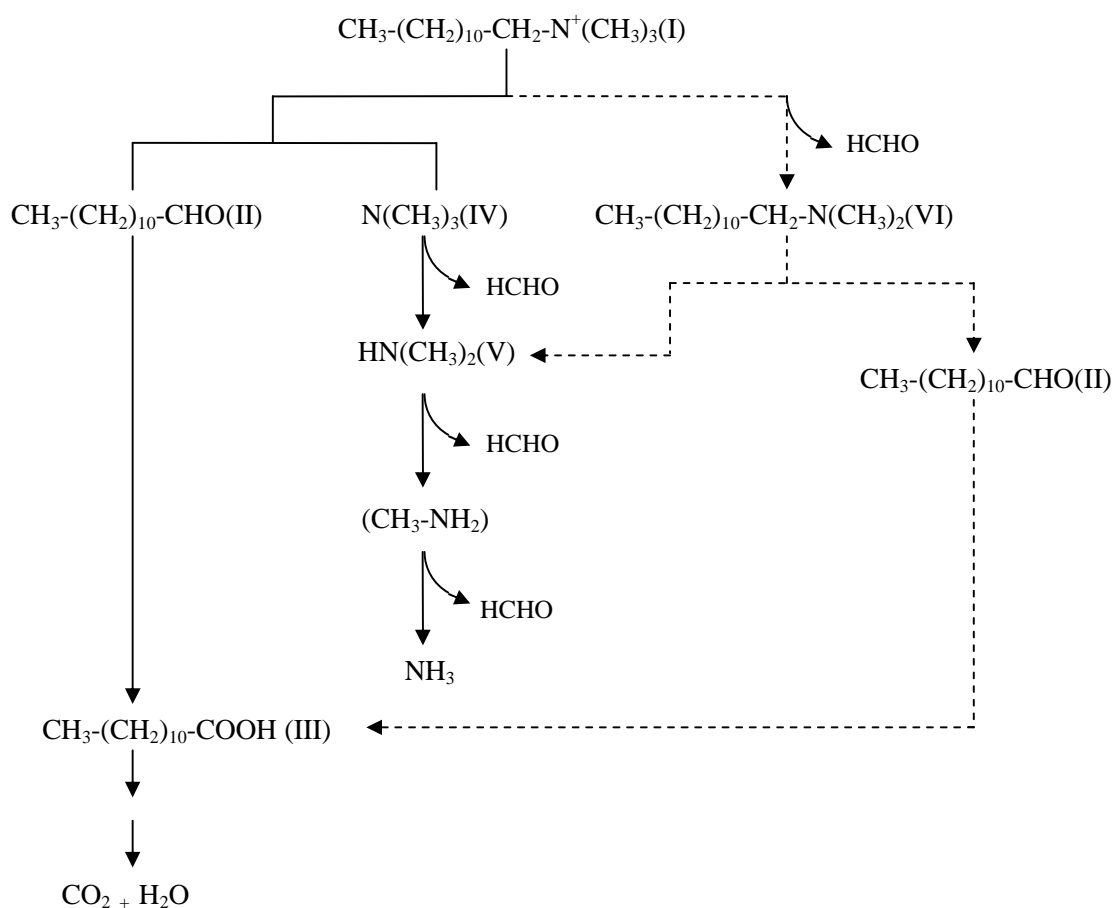


Figure 1.10 Proposed Pathways of *n*-dodecyltrimethylammonium Metabolism by *Pseudomonas* sp. strain 7-6. I, *n*-dodecyltrimethylammonium; II, *n*-dodecanal; III, lauric acid; IV, trimethylamine; V, dimethylamine; VI, *n*-dodecyldimethylamine. (Takenaka, et al., 2007).

The anaerobic degradation of tetradecylamine and other long-chain alkylamines by a newly isolated denitrifying bacterium was studied by Nguyen et al., (2008). The bacterium was isolated from a mixture of soil and activated sludge and was identified as Strain ZN6 representing *Pseudomonas stutzeri*, based on partial sequencing of the 16S rRNA gene. Strain ZN6 was a mesophilic, motile, Gram-negative rod-shaped bacterium, able to grow on a variety of compounds. When co-cultivated with a denitrifier *Castellaniella defragens* ZN3, anaerobic degradation under denitrifying of alkylamines by strain ZN6 was slightly faster. Strain ZN3 was a complete denitrifier, unable to convert tetradecylamine, and was copurified from the same enrichment culture as strain ZN6. They suggested that the pathway for the degradation of alkylamines in strain ZN6 started with C–N bond cleavage to alkanals and subsequent oxidation to the corresponding fatty acids.

Liffourrena et.al. (2008) studied the degradation of (TDTMA) by pure cultures of *Pseudomonas* strains in the presence of Lewis' acid. They observed that from the different strains of *Pseudomonas* screened, only *Pseudomonas putida* A ATCC 12633 grew with 50 mg/ l of TDTMA as the sole carbon and nitrogen source. They reported that *Pseudomonas putida* utilised TDTMA as its sole carbon and nitrogen source. The TMA produced in the initial step of the biodegradation was catalysed by a monooxygenase activity.

Ahmed et al., (2009) studied the aerobic degradation of 3-*N*-trimethylamino-1-propanol (homocholine) by a bacterium isolated from soil. The bacterium was identified as *Rhodococcus* sp. strain A2 based on its phenotypic features, physiological and biochemical characteristics and results of phylogenetic analysis. They observed that the washed cells of strain A2 completely degraded homocholine within 6 h, with the formation of several metabolites. Analysis of the metabolites using capillary electrophoresis, fast atom bombardment–MS, and GC–MS showed trimethylamine as a major product of metabolism, in addition to β -alanine, betaine and trimethylaminopropionaldehyde. They proposed that the degradation of homocholine in the isolated strain was through the oxidation of the alcohol group (-OH) to aldehyde (-CHO) and acid (-COOH) followed by the cleavage of C–N bonds which yielded trimethylamine and an alkyl chain.

Ahmed et al., (2009) isolated a gram-positive bacterium able to utilize 3-*N*-trimethylamino-1-propanol (homocholine) as sole source of carbon and nitrogen from soil. The strain was identified as *Arthrobacter* sp. strain E5 based on its phenotypic features,

physiologic and biochemical characteristics and phylogenetic analysis. Analysis of the products of degradation was by capillary electrophoresis and gas chromatography-mass spectrometry and showed trimethylamine, betaine and trimethylaminopropionaldehyde. They proposed a degradation pathway of homocholine in *Arthrobacter* sp. strain E5 to be via oxidation of alcohol group (-OH) to aldehyde (-CHO) and acid (-COOH), followed by the cleavage of C-N bond to yield trimethylamine and an alkyl chain.

Ahmed et al., (2010) isolated bacteria capable of degrading homocholine from soil. The bacteria were identified as *Pseudomonas* sp. strain A9 and *Pseudomonas* sp. strain B9b based on their phenotypic features and from phylogenetic analysis. Washed cells of strains A9 and B9b degraded homocholine completely within 6 h with formation of several metabolites. Analysis of the metabolites was by capillary electrophoresis, fast atom bombardment-mass spectrometry, and gas chromatography-mass spectrometry. Trimethylamine (TMA) was observed as a major metabolite beside β -alanine betaine and trimethylaminopropionaldehyde. They suggested that the possible degradation pathway of homocholine in the isolated strains was via oxidation of the alcohol group (-OH) to aldehyde (-CHO) and acid (-COOH), followed by the cleavage of the C-N bonds in β alanine betaine yielding trimethylamine and an alkyl chain.

In all these studies, two initial avenues of attack by microorganisms have been proposed: first, a hydroxylation of the alkyl chain terminus and second, fission of the C-alkyl-N bond. QACs have been reported to be ultimately biodegradable at low concentrations in river surface water and/or sediments (Larson and Vashon, 1983). All QAC biodegradation studies reviewed have utilized surfactants lacking benzyl groups except for the reports by Gawel and Huddleson, (1972) Hampton and Zatman, (1973), Fenger *et al.*, (1973) and Patrauchan and Oriel (2003), although benzyl groups are often included in commercial preparations to enhance bactericidal activity. Also, there are no reports on the isolation of QAC utilizing micro-organisms from the marine environment.

1.6 Potential Toxicity of QACs to Marine Organisms

The discharge of residual process chemicals in produced water to the marine ecosystem is of environmental concern owing to the effects of active ingredients such as quaternary ammonium compounds (QACs). Surfactants entering the environment via the discharge of effluents into surface waters have the potential to impact the ecosystem owing to their toxicity on organisms in the environment. Toxicity data from laboratory and field studies are essential in the assessment of the possible environmental risks from surfactants (Ying, 2006). In one study, Singh et al. (2002) tested seven surfactants for toxicity (immobility EC50-48 h) on six freshwater fish and observed that cationic surfactants were more toxic than anionic and nonionic surfactants. Immobility EC50-48 h is the median effective concentration of the surfactants that will induce the effect of immobility on the fresh water fish in 48 hrs. Also, in another study on toxic effects of Linear Alkyl Benzene Sulfonate (C₁₂LAS) and three quaternary alkylammonium chlorides on unicellular green algae *Dunaliella sp.*, Utsunomiya et al. (1997) observed the 24-h median effective concentrations (EC50-24 h) to be 3.5 mg/L for alkyl trimethyl ammonium chloride (TMAC), 18 mg/L for dialkyl dimethyl ammonium chloride (DADMAC) and 1.3 mg/L for alkyl benzyl dimethyl ammonium chloride (BDMAC).

In addition, Garcia et al. (1997) carried out acute toxicity tests on a freshwater flea *Daphnia magna* and *Photobacterium phosphoreum* for two families of monoalkyl quaternary ammonium surfactants namely alkyl trimethyl ammonium and alkyl benzyl dimethyl ammonium halides. They observed that 24-h immobilization EC50 on *D. magna* ranged from 0.13 - 0.38 mg/L for the six cationic surfactants whereas EC50 on *P. phosphoreum* ranged from 0.15 - 0.63 mg/L. Surfactants have relatively high sorption on sediment, sludge, and soil (Ying, 2006). Surfactants strongly associated with particulates or sediment; therefore, their biodegradation in sediment is useful in determining their fate in the environment (Ying, 2006). High concentrations of surfactants and their metabolites may affect organisms in the environment. The environmental risks caused by surfactants and their degradation products can be assessed based on the comparison of the predicted environmental concentration (PEC) and the predicted no-effect concentration (PNEC). Aquatic toxicity data for marine organisms are displayed in Table 1.3

Table 1.3 Aquatic Toxicity Data for QACs

| QACs | Species | Endpoint | References |
|----------------------|--|--|--------------------------|
| TMAC | <i>Dunaliella sp.</i> (green alga) | EC50– 24 h, 0.79 mg/L | Utsunomiya et al. (1997) |
| DADMAC | √ | EC50– 24 h, 18 mg/L | Utsunomiya et al. (1997) |
| BDMAC | √ | EC50– 24 h, 1.3 mg/L | Utsunomiya et al. (1997) |
| C ₁₆ TMAC | <i>Salmo gairdneri</i> (rainbow trout) | Immobilization EC50– 48 h, 1.21 mg/L | Singh et al. (2002) |
| | <i>Gambusia affinis</i> (mosquito fish) | Immobilization EC50– 48 h, 8.24 mg/L | |
| | <i>Carassius auratus</i> (goldfish) | Immobilization EC50– 48 h, 3.58 mg/L | |
| DTDMAC | <i>Salmo gairdneri</i> (rainbow trout) | Immobilization EC50– 48 h, 0.74 mg/L | Singh et al. (2002) |
| | <i>Gambusia affinis</i> (mosquito fish) | Immobilization EC50– 48 h, 7.91 mg/L | |
| | <i>Carassius auratus</i> (goldfish) | Immobilization EC50– 48 h, 2.37 mg/L | |
| DTDMAC | <i>Daphnia magna</i> (freshwater flea) | LC50– 48 h, 0.49mg/L NOEC–21 days, 0.38 mg/L | Lewis and Wee (1983) |
| DEEDMAC | <i>Daphnia magna</i> | Immobilization LC50– 24 h, 14.8 mg/L Growth NOEC– 21 days, mg/L | Giolando et al. (1995) |
| | <i>Pimphales promelas</i> (fathead minnow) | Growth NOEC– 35 days, 0.68 mg/L | |
| | <i>Selenastrum capricornutum</i> (algae) | Growth inhibition EC50– 96 h, 2.9 mg/L | |
| TMAC | <i>Daphnia magna</i> | Immobilization IC50– 24 h, 0.13– 0.38 mg/L | GarcõÂa et al. (2001). |
| BDMAC | <i>Daphnia magna</i> | Immobilization IC50– 24 h, 0.13– 0.22 mg/L | GarcõÂa et al. (2001). |

The endpoints show median Effect Concentration (EC50), Lethal Concentration (LC50), No Observed Effect Concentration (NOEC) and Inhibitory Concentration (IC50) and their effects on the different organisms. TMAC stands for Alkyl trimethyl ammonium chloride, DADMAC stands for Dialkyldimethylammonium chloride, BDMAC-Dodecylbenzyl dimethyl ammonium chloride, DTDMAC-Ditallow dimethyl ammonium chloride and DEEDMAC-Diethylester dimethylammonium chloride.

1.7 Research Outline and Objectives

This study is concerned with benzyl dimethyl hexadecyl ammonium chloride (BDHAC) (structure shown in Figure 1.1), and its degradation by heterotrophic bacteria isolated from marine sediments. All previous investigations carried out on the biodegradation of quaternary ammonium compounds (QAC) have centered on the metabolic fates of these substances in freshwater environments with the observation that QACs are biodegradable when present at low concentrations. However this research, investigating the aerobic biodegradation of BDHAC deals with the marine environment.

The objectives of the research are as follows:

1. to investigate whether bacteria isolated from marine sediment can degrade BDHAC;
2. to identify the strains of bacteria degrading the substrate;
3. to isolate individual strains and see whether they can use BDHAC as a sole source of carbon and energy;
4. to assess whether the individual strains isolated in (3) are able to degrade BDHAC at high concentrations and consequently, have potential for bioremediating sediment contaminated with BDHAC;
5. to identify the metabolites of bacterial degradation.

This research will contribute to an understanding of the impact of discharges of this chemical to the marine environment, in particular with respect to natural degradation rates

and formation of potentially hazardous metabolites. It will also show the potential of the isolated strains of bacteria in bioremediating BDHAC contaminated sites. This data will assist environmental risk assessment of oil production facilities and, following platform decommissioning, may provide a basis for a sediment remediation strategy.

CHAPTER 2

PRELIMINARY BIODEGRADATION STUDIES OF BENZYL-DIMETHYL-HEXADECYLAMMONIUM CHLORIDE (BDHAC) USING A MARINE BACTERIAL INOCULUM

An investigation into the biodegradation of BDHAC is presented here. The study reported in this chapter focuses on the isolation and identification of micro-organisms from marine sediment and their growth in enrichment cultures which contain BDHAC as sole carbon and energy source.

2.1 Introduction

Quaternary ammonium compounds (QACs) containing a long-chain alkyl group and/or benzyl group are cationic surfactants present in some industrial and household products (Garcia et al., 1997). They may be released and accumulated in aquatic environments where toxicity to organisms has been reported (Beveridge et al. 1998; Boething 1984; Tubbing and Admiraal 1991). A preliminary investigation of the ability of bacteria isolated from marine sediment to degrade BDHAC, a QAC and an active ingredient of many corrosion inhibitor formulations, was carried out. The objective was to see whether the bacteria could degrade BDHAC and, if so, to identify which strains of bacteria may be responsible.

2.2 Aim of the Study

This study investigated whether bacteria could be isolated from marine sediment that could degrade BDHAC at concentrations higher than that used in corrosion inhibitor formulations. The study, aims to answer the question; what type of bacteria can degrade the QAC in the marine environment?

2.3 Materials and Methods

Benzyltrimethylhexadecylammonium chloride (BDHAC) (Sigma Aldrich), ammonium chloride (NH_4Cl), potassium phosphate (K_2HPO_4), mercuric chloride (HgCl_2), methanol and marine broth were purchased from Fisher Scientific Company, United Kingdom. Nutrient agar was purchased from Oxoid Limited, Cambridge, United Kingdom. The reagents were analytical grade. BDHAC was the principal compound that was to be degraded by micro-organisms while other reagents were ingredients in the growth medium that would support the growth of bacteria. Mercuric chloride was used to inhibit the growth of bacteria in the control samples.

2.3.1 Glassware Cleaning Procedure

To minimise interference in analyses from contaminated glassware, a cleaning procedure was adopted according to the method of Henderson, (1999). The cleaning procedure was followed for all glassware used for sample preparation throughout this research. The procedure involved 1 hour washing of glassware at 50°C with Decon (Decon Laboratories Limited, Sussex, and UK.) and three subsequent rinses with tap water. This was followed by one rinse with 3% HCl , two rinses with tap water and one rinse with distilled water after which glassware was allowed to dry before a final rinse with dichloromethane (DCM). The DCM was left to evaporate.

2.3.2 Source of BDHAC-utilising Micro-organisms

Micro-organisms were isolated from inter-tidal sediment from the Firth of Forth, Scotland at Cramond Beach. Sediment was sampled with the aid of a plastic scoop and was placed in a four litre plastic bucket, a lid fitted and the bucket transferred to the laboratory at ambient temperature. The sediment was stored at room temperature and was later used as a source of bacteria in the preparation of inocula. The sampling site was selected because the area is exposed to a number of pollutant sources (an industrial area near a major city) and therefore would have a diverse bacterial population.

Inocula were created by adding 3.32 g (wet weight) of sediment to five 50 ml flasks containing seawater from St Andrews, Scotland. The seawater was autoclaved so that the sediment would be the only source of bacteria in the inocula prepared. The flasks containing the sediment solution were then agitated constantly for 5 minutes to help mix the bacteria within the sediment into suspension. The flasks were then labelled 1-5 and left for 30 minutes to allow the sediment to settle and the bacteria to move into suspension. This method was according to Diaz (2000).

2.3.3 Preparation of Growth Media

2.5 litres of clean seawater was mixed with 2.5g of ammonium chloride (NH_4Cl) and 1.25g of potassium phosphate (K_2HPO_4), giving a resultant concentration of 1 and 0.5g/l of the salts respectively. These chemicals were nutrients that would support the growth of the bacteria in the biodegradation experiment. These salt concentrations were as specified by the method of Diaz (2000). The medium was subsequently split into 100 ml portions in 250 ml Erlenmeyer flasks, sealed with foam bungs and then autoclaved.

2.3.4 Preparation of Test Samples and Controls

5 g BDHAC was dissolved in 100 ml of methanol in a 100 ml volumetric flask (**Standard 1**) and 100 μl (5 mg BDHAC) was added to five 250 ml Erlenmeyer flasks each with 100 ml of medium. Thereafter, 1ml of medium was carefully removed from the test flasks using a pipette and replaced with 1ml of bacterial inoculum that was prepared earlier (Section 2.3.2), and the flasks were labelled DP1-5. Two control flasks, Control 1 and 2 was set-up to run for the duration of the experiment and had the same content as the test flasks but without the addition of 1ml of bacterial inoculum. Instead, 1ml of 1% HgCl_2 and 10ml of 1% HgCl_2 were added to the flasks, respectively. Mercuric chloride was added to the control samples to prevent any bacterial activity (Diaz, 2000).

Altogether 5 tests and 2 controls samples were made up for the biodegradation experiment. The flasks were placed on a rotary shaker at 25^0C at 120 revolutions per minute (rpm) for 43 days. Shaking was used to provide a continuous supply of oxygen for the

growth of the bacteria.

2.3.5 Isolation of BDHAC Utilising Micro-organisms

The isolation of micro-organisms (such as bacteria) from complex mixed cultures and their cultivation in a pure culture is an essential prerequisite for their precise identification and characterization. Several different processes are used for this purpose (Fröhlich and König, 1999). Most microbial isolation procedures involve some form of separation to obtain microbial cells. The most common approach is the use of an agar-based medium for primary isolation, with streak dilution and spread plating to produce single colonies which are derived from individual micro-organisms. Samples are often diluted before isolation, so that a small number of individual microbial cells are transferred to the growth medium (Alexander, 1999)

For the isolation of micro-organisms for this research, plates were prepared by dissolving 18.7g of marine broth (Difco, Voigt Global Distribution Inc, USA) and 7.5g of nutrient agar (Oxoid, Basingstoke, UK) in a 500ml flask with distilled water, and autoclaving at 121°C for 15 minutes, before cooling in a water bath. Empty plates were kept under ultraviolet (UV) light for 5 minutes to sterilise them, after which marine agar solution was poured into the plates and allowed to set. Saline solution for serial dilution of test samples was prepared by dissolving 17g of NaCl (Fisher, Loughborough, UK) in 2 litres of distilled water and transferring 9.0 and 9.9 ml of the resulting solution to test tubes using a pipette.

On days 28, 33, 38 and 43 of incubation, 1ml and 100µl samples were withdrawn from flasks DP1-5 and Control 1 and were serially diluted to obtain dilutions of 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-6} , 10^{-8} and 10^{-10} . 100µl of 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} and 10^{-6} , 10^{-8} and 10^{-10} dilutions of cultures in the test and control flasks were plated out (two plates per dilution) using glass beads to evenly spread the culture solution on the plates. The plates were incubated at 25°C to isolate bacterial colonies. On day 43, after samples were removed from the test flasks and the control for colony isolation, quantification of residual BDHAC in the flasks was carried out.

2.3.6 Characterization of Bacterial Isolates

Streaking a plate is a basic skill used in the isolation of a cell culture and maintaining stock cultures and the aim is to achieve single colonies on the agar plate. These colonies are derived from single cells. A sterile inoculating loop is used to streak micro-organisms over the surface of a medium thereby diluting the sample. A streak dilution plate with single colonies all of the same type confirms the purity of the strain (Alexander, 1999). Isolates from experimental flasks on days 28, 33, 38 and 43 were streaked to purity on fresh marine agar plates and single cultures obtained were subjected to some identification tests. The tests were micromorphology, motility, catalase, oxidase and 16S-rDNA sequencing.

Physical Characteristics

Isolates were investigated to determine their physical characteristics. The tests performed on the isolates are discussed below.

Micromorphology: Gram staining is used to differentiate bacterial species into two large groups (Gram-positive and Gram-negative) based on the physical and chemical properties of their cell walls (Ryan and Ray, 2004). The technique is used as a first step to determine the identity of a particular bacterial sample (Madigan et al., 2004). The procedure was performed as follows. A smear of each bacterial isolate was heat-fixed on a clean grease-free slide, crystal violet stain was flooded on the slide for 60sec, rinsed under a slow running tap and washed with Lugol's iodine solution for 60sec and then drained off with water gently under the tap. 70 % ethanol solution was then used to decolorize the stain for 10sec. The sample was then rinsed under tap water and flooded with safranin for 60sec to counter stain, washed with water and blotted dry. The slide was then examined using a compound microscope with an oil immersion objective lens (x1000) for identification. The species that retain the crystal violet (blue or purple) are termed Gram-positive while those stain red or pink are described as Gram-negative (Gerhardt *et al.*, 1981).

Motility Test: The test demonstrates motility of flagellated bacteria as shown by the “hanging drop” technique (Collins and Lyne, 1984). In this test, 24 hour-old cultures of the bacterial isolates grown on plates were used. A little vaseline was applied around the hollow in a cavity slide, and a loopful of culture was transferred to the centre of the cavity and covered firmly with a cover slip by pressing down gently and firmly so that the drop of culture was laid in the form of a hanging drop. When viewed under x40 objective lens, motile cells darting across the field of view indicated a positive result.

Biochemical Testing: Biochemical testing was carried out to check some metabolic properties of the different isolates of bacteria.

Catalase Test: This test is used to differentiate organisms that have the ability to produce the enzyme catalase. This enzyme is responsible for protecting bacteria from hydrogen peroxide (H_2O_2) accumulation, which can occur during aerobic metabolism. If hydrogen peroxide accumulates, it becomes toxic to the organism. Catalase breaks H_2O_2 down into water and O_2 . In this test a drop of 3 % H_2O_2 (Sigma Aldrich, United Kingdom) was added to a streaked plate from a colony that was incubated for 24 hr and observed for instantaneous production of gas bubbles or effervescence. The presence of a bubbling reaction indicated a positive result (Collins and Lyne, 1984).

Oxidase Test: This test identifies cytochrome c oxidase, an enzyme found in obligate aerobic bacteria. Obligate aerobic bacteria are those bacteria that cannot survive without oxygen. The test was carried out according to the method of Cruickshank et al., (1975) as follows. A strip of filter paper was soaked with a little freshly prepared 1% solution of tetramethyl-*P*-phenylene-diamine dihydrochloride (BDH). Colonies on marine agar (Difco) were then picked with the edge of a sterile clean slide and rubbed on the soaked strip of filter paper. Intense deep-purple coloration appearing almost immediately indicated a positive result.

Identification of Strains by 16S rDNA Sequencing: Some bacteria are difficult to identify with phenotypic identification methods. A phenotype is any observable characteristic or

trait of an organism: such as its morphology, development, biochemical or physiological properties, or behaviour. Therefore 16S ribosomal DNA (rDNA) - based identification of bacteria potentially confirms findings from the phenotypic characterisation methods (Drancourt and Raoult 1999). Tentative identification of bacterial isolates was made by means of partial sequence analysis of the 16S rDNA gene (Drancourt and Raoult 1999).

Genomic DNA Extraction: Genomic DNA was extracted using a modification of the protocol described by Brunt and Austin (2005). The bacterial cultures were grown in marine broth (Difco) on a rotary shaker (120rpm) for 24 h at 28°C, and harvested by centrifugation at 3000 x g for 10 minutes at 4°C. The cells were washed twice and re-suspended in 500 µl (0.5 ml) PBS. The cells were then lysed with lysis buffer, and the DNA extracted using a DNA extraction Kit (Qiagen) following the manufacturer's protocol for extraction of genomic DNA.

Amplification of 16SrDNA by PCR: In a sterile 0.2 ml amplification tube (Greiner), a PCR reaction mixture was assembled by adding components in the following order: 25 µl of biomix buffer solution (Bioline) containing 1.0 unit *Taq* DNA polymerase (Amersham Bioscience); 1.0 µl of forward and reverse primers; 1.0 µl of template DNA; 2.0 µl of 2.0 mM stock solution of four dNTPs, 1.0 µl of 1.5 mM MgCl₂, 2.0 µl of 32 mM (NH₄)₂SO₄, 1.0 µl of 125 mM Tris- HCl and 1.0 µl of 0.02 % (v/v) Tween 20, and 15.0 µl sterile MilliQ (Millipore) water to achieve a final volume of 50 µl.

Two bacterium specific primers were used to amplify approximately 1500 base pairs of rDNA. The oligonucleotide primers used to amplify the target gene were the forward primer 27F (5'AGAGTTTGATCMTGGCTCAG-3') and the reverse primer 685r³ (5'TCTRCGCATTYCACCGCTAC-3') (Lane, 1991 obtained from MWG Biotech, Cork, Ireland). The nucleic acids were amplified using an icycler (Bio-Rad) and the denaturation, annealing and polymerization (extension) times were as follows: initial denaturation at 96°C for 4 minutes, followed by 30 cycles of denaturation at 95°C for 30 s; annealing at 55°C for 30 s; polymerization (extension) at 72°C for 1 minute, and a final extension at 72°C for 7 minutes. Controls, without template DNA, were included in the

amplification process alongside test samples. The test reaction mixtures were stored at -20 °C until needed.

Agarose Gel Electrophoresis of DNA: For the assessment of DNA extracted from the bacterial isolates, agarose gel electrophoresis was carried out using the Horizon 58 gel electrophoresis apparatus (Gibco, BRL). 1 g of agarose (Integra Bioscience) was dissolved in 100 ml of Tris-borate-EDTA (TBE) buffer by heating in a microwave oven. After dissolving, the solution was allowed to cool to 55°C, before 1.0 µl of 10mg/ml ethidium bromide (Sigma Aldrich, UK) was added. The gel solution was thoroughly mixed and poured into a taped gel casting tray with a 14 well integrated comb, and left to cool and solidify in a cool room. After this, the gel was then transferred and electrophoresis buffer–Tris-borate EDTA (TBE) was poured to cover the agarose gel to the top, followed by careful removal of the comb to reveal the 14 sample wells.

Gel loading buffer was prepared as follows:

| | |
|---|-----------|
| 0.25 % (w/v) bromophenol blue | 0.0125 ml |
| 0.25 % (w/v) ethylene cyanol | 0.0125 ml |
| 25 % (v/v) glycerol | 1.25 ml |
| 1 % (w/v) sodium dodecyl sulphate (SDS) | 0.05 ml |
| 0.5M EDTA, pH 8 | 1.5 ml |
| Sterile water | 2.25 ml |

For all cases 10-20 µl of sample DNA was mixed with 2 µl of gel loading buffer before loading the wells. A marker was loaded on one of the wells for a comparative molecular weight determination of the DNA. A blank (without DNA sample) was pipetted into one

other well for use alongside the test samples. Electrophoresis was then carried out by connecting the electrophoresis chamber to the source of power and allowed to run for 2 h at 93 V or for an unspecified length of time until the tracking dye had diffused to the bottom of the agarose gel. Following electrophoresis, the DNA banding patterns on the agarose gel were immediately observed with an ultra violet (UV) Trans illuminator (UV Products). Photographic records were taken using a gel documentation unit (Amersham Bioscience).

Purification of PCR Products: PCR products were purified using a protocol from (Promega, USA). Approximately 1µg of each fragment was suspended in 200µl of TE and precipitated at -20°C in 100µl of absolute ethanol and 20 µl of sodium acetate (3M, pH 5.2). Fragments were rinsed twice with 300 µl of ethanol (70%) and then dried at 37°C for 1h. 500ng of each PCR product were digested using *EcoRI* (Promega, USA) in a total volume of 20 µl. The restriction fragments were resolved by electrophoresis on a 1% agarose gel.

Sequencing of Purified PCR Products: The purified PCR products were sequenced in both directions; forward and reverse using 20 µl reaction mixtures containing 4 µl of big dye deoxy Terminator Cycle Sequencing Kit (Applied Biosystems), 4 µl of x 5 sequencing buffer, 1 µl of either forward or reverse primer (5p mol/µl), 2 µl of purified PCR product and 9 µl of sterile MilliQ (Millipore) water. Cycle sequencing reactions were accomplished by initial denaturation at 98°C for 5 minutes, followed by 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s and extension at 60°C for 4 minutes. The reaction products were purified by the addition of 40 µl of 75% (v/v) isopropanol (Sigma), and then incubated at room temperature for 10 minutes to allow for precipitation of the DNA. Samples were then centrifuged at 10,000 x g for 20 minutes to pellet the DNA. The overlying supernatants were removed, and the resultant pellets were washed three times with 125 µl of 70 % (v/v) isopropanol to remove excess dyes. The resultant samples were dehydrated at 90°C for 1 minute. Purified templates (dehydrated samples) were re-suspended in 25 µl of template suppression reagent (TSR, Applied Biosystems), heated at 95°C for 2 minutes, and were analysed using an ABI Prism 310 Genetic Analyser (Applied Biosystems). The resulting chromatograms were examined using Chromas Pro Version

1.21 software (Technelysium), and forward and reverse sequences were compared and corrected using GENETYX version 7.0.3 packed programs (Software Development Co.). The resulting 16S rDNA gene sequences were compared in a Basic Local Alignment Search Tool (BLAST) search with those in the National Library of Medicine database. BLAST finds regions of local similarity between sequences. It compares nucleotide (or protein) sequences to sequence databases and calculates the statistical significance of matches.

2.3.7 Quantification of Residual BDHAC after Enrichment

After enrichment, it was important to quantify the amount of residual BDHAC in the flasks in order to assess the extent of degradation by bacteria. Quantification of residual BDHAC in the different flasks was performed on day 56 after initial inoculation to determine if the strains were able to utilize it during the incubation period. This was carried out by Colorimetric Dye Binding Assay (CDBA) and Electrospray Ionisation Tandem Mass Spectrometry (ESI-MS/MS). 50 ml samples from five test and one control flasks were extracted with 50ml of dichloromethane (DCM) and the extracts were collected in 50ml volumetric flasks. The 50ml DCM extracts were further divided into two equal parts, 25ml each, and were labelled samples A and B. Sample A (25ml) was analysed by ESI-MS/MS while sample B (25ml) was analysed by CDBA following the method of Waters and Kupfer, 1976).

Preparation of a Calibration Curve for CDBA Analysis: A calibration curve was prepared using a spectrophotometer (Ultraspec 1000E manufactured by Pharmacia Biotech, Cambridge, England.). This was carried out prior to analysis of samples by CDBA. For the calibration, 50mg of BDHAC was dissolved in methanol in a 50ml volumetric flask resulting in 1mg/ml or 1000mg/l standard solution (**Standard 1**). 1.0, 0.5, 0.25 and 0.1 ml of **Standard 1** were added to 4 different 50ml volumetric flasks with growth media (see section 2.3.3) and the resulting concentrations were 20, 10, 5 and 2mg/l. Sample solutions with these concentrations were extracted with 50ml DCM and the DCM extracts were placed in 50ml volumetric flasks and was blown to dryness with a gentle stream of nitrogen. The

volumetric flasks were then filled with sea water up to the 50ml mark to dissolve residual BDHAC. The resulting solutions were then transferred to 250ml separating funnels where 50ml of 9.1 % buffer (sodium phosphate, (Fisher), pH 8.2 and 1.0ml 0.5 % methyl orange, was added and swirled to mix before 10ml chloroform previously saturated with phosphate buffer was added using a pipette. Saturation of the chloroform by the buffer was achieved by mixing 100ml of phosphate buffer with 50ml of chloroform in a 250ml separating funnel and chloroform that settled to the bottom of the funnel was completely drained out. The separating funnel containing a mixture of BDHAC solution, buffer, methyl orange and saturated chloroform was vigorously shaken for 10min and allowed to settle for about 15min. The lower chloroform layer in the funnel was removed and was analysed spectrophotometrically; a small volume was poured into a cuvette and absorbance was read at 450nm using chloroform as the reference (Waters and Kupfer, 1976). From these absorbance values, a calibration curve was produced by plotting absorbance value against BDHAC concentration. The basic principle involved in this technique is the formation of a complex between methyl orange and BDHAC and the subsequent extraction of this complex by the chloroform.

Quantification of Residual BDHAC by CDBA: Sample B (25ml of DCM extract) (as described in section 2.3.7) was placed in a 50ml volumetric flask and was blown to dryness with a gentle stream of nitrogen. The volumetric flask was then filled with sea water up to the 50ml mark (**Solution 2**) to dissolve residual BDHAC. Solution 2 was transferred to a 250ml separating funnel and the same process described in section 2.3.7 was carried out.

BDHAC Recovery Analysis: To account for losses of BDHAC by adsorption to glassware, a recovery experiment was performed. For the experiment, three 250ml Erlenmeyer flasks labelled A, B and C were filled with 100ml of growth medium (see section 2.3.2) and to flasks A, B and C 100 μ l of 5% BDHAC stock standard was added to give concentrations of 5mg/100ml or 0.05mg/ml. The flasks were left to sit for 24 hr before 50 ml solution was removed from the flasks and used for the recovery experiment. These volumes were extracted with 50ml of DCM in a 250ml separating funnel and 25ml of the DCM extract was transferred to a 50ml volumetric flask using a pipette. This sample was blown to

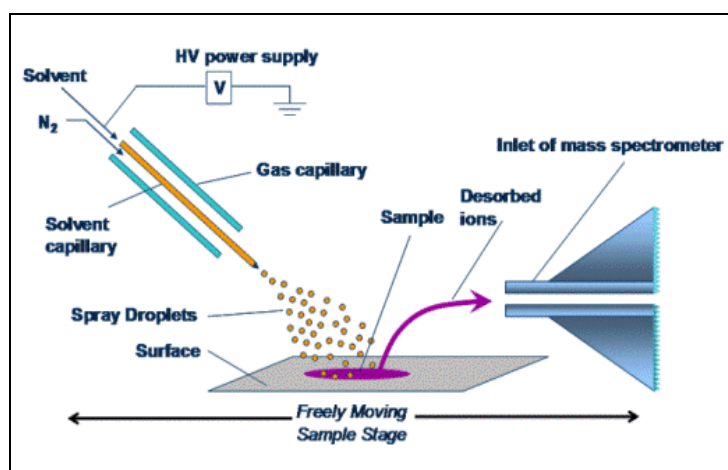
dryness with a gentle stream of nitrogen, and BDHAC residues in the flasks were taken up in seawater and made up to the 50ml mark before analysis by colorimetric dye binding assay was carried out.

Electrospray Ionisation Mass Spectrometry (ESI-MS): Electrospray ionization (ESI) is a technique used in mass spectrometric analysis to produce ions. It is very useful in the production of ions from macromolecules owing to its ability to overcome the propensity of these molecules to fragment when ionized. Electrospray ionisation tandem mass spectrometry (ESI-MS) is an important technique in environmental analysis as it provides a sensitive, robust, and reliable tool for studying unknown compounds in samples (Ho et al., 2003). With additional separation capabilities of tandem mass spectrometry (mass spectrometry (MS) in a series, commonly denoted as MS/MS), complicated sample analysis can be much simplified (Ho et al., 2003).

In a typical tandem mass spectrophotometer, there is a tandem quadrupole system where three quadrupoles are set up in a linear fashion, often called “triple-quad”. The analyte ion of interest (usually called the precursor ion) is mass-selected by the first quadrupole (Q1) and allowed to collide with argon (collision gas) in a second quadrupole collision cell (Q2), where the precursor ions are activated by collision and undergo further fragmentation. This process is known as collision-induced dissociation (CID). The daughter ions resulting from CID are related to the molecular structure of the ions and can be monitored by a third quadrupole mass analyser (Q3) providing structural information of the molecular ions. This tandem system is commonly denoted as MS/MS (Bruins, 1998).

Electrospray ionization tandem mass spectrometry (ESI-MS) uses electrical energy to aid the transfer of ions from solution into the gaseous phase before they are subjected to mass spectrometric analysis (Ho et al., 2003). The transfer of ionic species from solution into the gas phase by ESI involves three steps: (a) the dispersal of a fine spray of charge droplets, followed by (b) solvent evaporation and (c) ion ejection from the highly charged droplets tube, which is maintained at a high voltage (e.g. 2.5 – 6.0 kV) relative to the wall of the surrounding chamber. During analysis, a mist of highly charged droplets with the same polarity as the capillary voltage is generated and the application of a nebulising gas such as nitrogen, shears around the eluted sample solution and enhances a higher sample

flow rate (Ho et al., 2003). The charged droplets, generated at the end of the electrospray tip, pass down a pressure gradient and potential gradient toward the analyser region of the mass spectrometer and with an elevated ESI-source temperature and/or another stream of nitrogen drying gas, the charged droplets are continuously reduced in size by evaporation of the solvent, leading to an increase of surface charge density and a decrease of the droplet radius. Finally, the electric field strength within the charged droplet reaches a critical point at which it is kinetically and energetically possible for ions at the surface of the droplets to be ejected into the gaseous phase. The emitted ions are sampled by a sampling skimmer cone and are then accelerated into the mass analyser for subsequent analysis of molecular mass and measurement of ion intensity (Bruins, 1998). Figure 2.1 shows a schematic of an ESI-MS source.



Source: Prosolia Incorporated.

Figure 2.1 Schematic of an ESI-MS Source

In this research, a Quattro Ultima triple quadrupole tandem mass spectrometer equipped with an electrospray interface from Micromass (Manchester, UK) was employed. Argon was used as the collision gas and nitrogen as the nebulizer gas. The ions were detected using Multiple Reaction Monitoring (MRM) in the positive ion mode. In MRM, the first analyzer allows only a single mass through and the second analyzer allows for multiple user defined fragment ions. Capillary voltage was set to 3.5 KV. Source and desolvation

temperatures of 120°C and 250°C, and cone and desolvation nitrogen gas flows of 80 L/h and 400 L/h, were selected. BDHAC was quantitatively analysed with a C14 QAC used as an internal standard. This was achieved by integrating the respective analyte and internal standard QAC peaks, determining the ratio of analyte peak area to internal standard peak area, and plotting the analyte/internal standard area ratio versus the QAC analyte solution concentration to obtain a calibration curve. (Zhang et al., 1997 and Ferrer and Furlong, 2001)

Preparation of Calibration curve for the Quantification of BDHAC by ESI-MS/MS: For the calibration, 63 mg of BDHAC (also referred to as C16 Quat or Quat in this thesis) was weighed into a 100ml volumetric flask. The flask was then filled to the mark with 90:10 methanol/water. The concentration of the resulting solution was 630mg/l of BDHAC (C16 Quat stock standard). 69mg of C14 Quat (Internal Std) was weighed into a 100ml flask and the volume was made up to the 100ml mark with 90:10 methanol/water. The concentration of the resulting solution was 690mg/l. 3.5ml of 630µg/ml C16 Quat was later made up to the mark with 90:10 methanol/water in a 10ml volumetric flask and a concentration of 220.5µg/ml (**Standard A**) was obtained. 4ml of 630µg/ml C16 Quat was made up to the mark with 90:10 methanol/water in a 10ml volumetric flask and a concentration of 252µg/ml (**Standard B**) was obtained. 1ml of 690µg/ml C14 Quat was made to the mark with 90:10 methanol/water in a 10ml volumetric flask and the resulting concentration was 69µg/ml. (**Standard C**).

From the stock standards of 220.5 µg/ml C16 Quat (**A**) and 69 µg/ml C14 Quat (internal standard) (**C**), serial dilutions were made as follows: 5ml of A was added to a 10ml volumetric and made up to the mark with 5ml of C. The resulting concentrations were 110.25 µg/ml of A and 34.5 µg/ml of C. 5ml of A (220.5 µg/ml C16 Quat) was added to a 10ml volumetric and made up to the mark with 5ml methanol and the resulting solution was serially diluted in 5ml of methanol to give concentrations of 55.125, 27.563, 13.781, 6.891 and 3.4453, 1.7227 and 0.8613 µg/ml of C16 Quat. From the last solution of 0.8613µg/ml, 5ml was discarded into a waste solvent bottle and 5ml of 69 µg/ml C14 Quat internal standard was added to all the flasks, resulting in a serial dilution containing 34.5 µg/ml C14 Quat. Similar serial dilutions as described above were prepared using Standard B i.e. 252

$\mu\text{g/ml}$ C16 Quat and the resulting concentrations from the dilutions were 126, 63, 31.5, 15.75, 7.825, 3.9375, 1.968, 0.9844, 0.4921, 0.2461, and 0.1231 $\mu\text{g/ml}$. 5ml of the most dilute solution was discarded from each solution into a waste solvent bottle and 5ml of 69 $\mu\text{g/ml}$ C14 Quat internal standard was added to all the flasks, resulting in serial dilutions containing 34.5 $\mu\text{g/ml}$ C14 Quat. This was carried out to obtain further dilutions for the standard values obtained earlier. 10 μl of the standards were injected into the mass spectrometer and were analysed in duplicate by the ESI-MS process. The peaks were quantified and a calibration curve was produced by plotting the peak ratio of BDHAC to C14 (internal standard) against concentration.

Quantification of Residual BDHAC by ESI/MS-MS: After the calibration curve was obtained, quantification of residual BDHAC in samples from the degradation experiment was performed. In this experiment, Sample A (25ml) (as described in section 2.3.7) was gently blown to dryness with a gentle stream of nitrogen and was made up to the mark (25ml) with methanol/water 90:10. Samples were treated as described for the calibration experiment with the addition of the same amount of C14 internal standard. The resulting solution was used for the quantification of residual Quat in solution. This was achieved by injecting 10 μl of the test solution into the mass spectrometer using a closed loop injector and peaks produced were quantified; the equation of the line from the calibration curve that was obtained from section (2.3.7) was used in the computation of percentage degradation values.

2.4 Results

2.4.1 Isolation of BDHAC-utilising Micro-organisms

Plates incubated with samples from the different flasks collected on day 28 had no colonies growing on them at dilutions of 10^{-2} , 10^{-4} , 10^{-6} , 10^{-8} , and 10^{-10} while plates that were incubated with samples collected on day 33 showed growth of colonies. Plates with samples from flask DP3 were seen to have amber coloured colonies at $\times 10^{-2}$ and $\times 10^{-3}$ dilutions. These plates were labelled DP3⁻² and DP3⁻³. Plates with culture from flask DP2 with

PRELIMINARY BIODEGRADATION STUDIES

dilution of $\times 10^{-3}$ and $\times 10^{-4}$ had 121 and 110 colonies growing on them. These colonies were white and transparent. These plates were labelled DP2⁻³ and DP2⁻⁴. On plates incubated with samples collected on day 43, amber colonies were observed on a plate with culture from flask DP1 at a dilution of $\times 10^{-2}$. This was labelled DP1⁻². In addition, plates with culture from flask DP4 had white transparent colonies at $\times 10^{-2}$ and 10^{-3} dilutions while plates with cultures from flask DP5 and the control showed growth of colonies at dilutions of $\times 10^{-2}$, 10^{-3} ; $\times 10^{-2}$ and 10^{-4} respectively. In general two different colony types were seen growing on the plates from these flasks except for flask DP1. Also, growth of colonies on the plate with $\times 10^{-2}$ diluted cultures from Control Flask 1 was surprising as inoculum was not added and the flask contained 1ml of 1% HgCl₂ which has been reported to kill bacteria according to a study by Diaz (2000). After the cell count procedures, flasks DP1-5 and the control were stored at 4°C in a cold room, and on day 56, analysis for the quantification of residual BDHAC in the flasks was performed.

Table 2.1: Viable Colony Counts and CFU/ml Values from Samples during 43days Incubation.

| Isolates | Cell Counts | CFU/ml |
|---------------------|-------------|--------------------|
| DP1 ⁻² | 160 | 1.6×10^5 |
| DP2 ⁻³ | 121 | 1.21×10^6 |
| DP2 ⁻⁴ | 110 | 1.10×10^7 |
| DP3 ⁻² | 165 | 1.65×10^5 |
| DP3 ⁻³ | 132 | 1.32×10^6 |
| DP4 ⁻² | 166 | 1.66×10^5 |
| DP4 ⁻³ | 125 | 1.25×10^6 |
| DP5 ⁻² | 175 | 1.75×10^5 |
| DP5 ⁻³ | 153 | 1.53×10^6 |
| CONT1 ⁻² | 60 | 6.0×10^4 |

CFU= (Cell Count $\times 10^x$) $\times 10$ for 1ml of sample where 10^x is the dilution factor.

Table 2.1 illustrates viable colony counts from enrichment cultures in test flasks DP1-5 and Control 1. The highest number of colonies (175) was observed on plate DP5⁻³ i.e. the $\times 10^{-3}$ dilution of culture from test flask DP5. However the lowest cell count (60) was observed on the plate with culture from Control flask 1 diluted $\times 10^{-2}$ i.e. (CONT1⁻²). The results obtained for the viable colony count revealed some unexpected findings in that successive dilutions were shown to yield almost the same number of colonies. However, the important thing was that colonies were isolated following 43 days growth with BDHAC, and these were available for further analysis.

2.4.2 Characterisation of Bacterial Isolates

Table 2.2: Physiological Characteristics and Tests on Bacterial Strains

| Source | Isolates * | Catalase Test | Oxidase Test | Gram Stain | Motility Test | Shape |
|---------------------|-------------------------------|---------------|--------------|------------|---------------|------------------|
| DP1 ⁻² | <i>Bacillus subtilis</i> | + | + | Gram +ve | + | Rods |
| DP2 ⁻³ | <i>Micrococcus luteus</i> | ++ | + weak | Gram +ve | | Coccus |
| DP2 ⁻⁴ | <i>Bacillus niabensis</i> | ++ | + weak | Gram +ve | | Rods |
| DP3 ⁻² | <i>Thalassospira sp.</i> | + | + | Gram -ve | + | Rods |
| DP3 ⁻³ | <i>Rhodospirillaceae</i> | + | + | Gram - ve | + | Rods |
| DP4 ⁻² | <i>Staphylococcus equorum</i> | + | ++ | Gram +ve | + | Coccus |
| DP4 ⁻³ | <i>Sporosarcina sp.</i> | ++ | + | Gram -ve | + | Filamentous rods |
| DP5 ⁻² | <i>Thalassospira sp</i> | + | + | Gram -ve | + | Rods |
| DP5 ⁻³ | <i>Micrococcus luteus</i> | + | + | Gram +ve | - | Coccus |
| CONT1 ⁻² | <i>Actinobacterium</i> | + | | Gram +ve | - | Rods |

* Identifications from 16Sr-DNA gene sequencing (section 2.4.3)

Isolates from experimental flasks on days 28, 33, 38 and 43 were streaked to purity on fresh marine agar plates and single colonies obtained were subjected to some identification tests and the results of the tests are summarised in Table 2.2.

The first column on the table shows the source of the isolates i.e. cultures in test flasks DP1-5 and Control 1 and the different dilution factors for example DP4⁻² showing $\times 10^{-2}$ dilution of culture contained in test flask DP4. Generally, the isolates were motile except for isolate DP2⁻³ and DP2⁻⁴, DP5⁻³ and CONT1⁻². Also, all the isolates were oxidase and catalase positive. Almost all the isolates shown on the table were Gram-positives except isolates DP3⁻², DP3⁻³, DP4⁻³ and DP5⁻² that were Gram-negatives. Generally, the colonies that grew on the plates were not similar.

2.4.3 Identification of Bacterial Isolates by 16SrDNA Gene Sequencing

The Ten isolates were tentatively identified by partial sequencing of the 16S ribosomal DNA gene (Figure 2.2 and Table 2.3). Isolate DP1⁻² showed 100 % identity to *Bacillus subtilis*, DP2⁻³ and DP2⁻⁴ showed 99 and 100 % identities to *Micrococcus luteus* and *Bacillus niabensis* respectively. Also DP3⁻² and DP3⁻³ showed 96-100% identities to *Thalassospira* sp. and *Rhodospirillaceae*. DP4⁻² and DP4⁻³ showed 100% identities to *Staphylococcus equorum* and *Sporosarcina* sp. while DP5⁻², DP5⁻³ and CONT⁻¹ showed 99, 99 and 100% identities respectively to *Thalassospira* sp., *Micrococcus luteus* and *Actinobacterium* according to the National Center for Biotechnology Information (NCBI) BLAST database. Results obtained from the sequencing of the 16S ribosomal DNA gene of the isolates were consistent with those of the phenotypic characterisation tests (section 2.3.6 and 2.4.2).

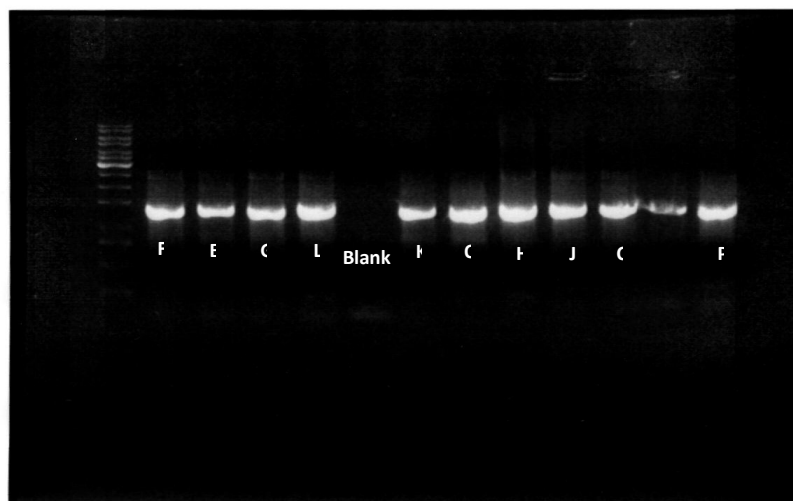


Figure 2.2: Agarose gel electrophoresis of PCR-amplified 16S rDNA segments of ten isolates

Figure 2.2 describes the agarose gel electrophoresis of PCR-amplified 16S rDNA segments of the 10 isolates. The figure shows sample wells which differentiates the blank from the actual samples that contains DNA. Each lane shown on the figure contains amplified PCR products. The alphabets represent the different isolates and the empty well is the blank sample.

Table 2.3: 16S-rDNA Analysis on Bacterial Isolates

| Bacteria Isolates | Symbol | Organism | % Identity |
|---------------------|--------|-------------------------------|------------|
| DP1 ⁻² | F | <i>Bacillus subtilis</i> | 100% |
| | F [r] | <i>Bacillus subtilis</i> | 100% |
| DP2 ⁻³ | E | <i>Micrococcus luteus</i> | 99% |
| | E [r] | <i>Micrococcus luteus</i> | 100% |
| DP2 ⁻⁴ | G | <i>Bacillus niabensis</i> | 100% |
| | G [r] | <i>Bacillus niabensis</i> | 97% |
| DP3 ⁻² | L | <i>Thalassospira sp.</i> | 100% |
| | L [r] | <i>Thalassospira sp.</i> | 96% |
| DP3 ⁻³ | K | <i>Rhodospirillaceae</i> | 100% |
| | K [r] | <i>Rhodospirillaceae</i> | 99% |
| DP4 ⁻² | Q | <i>Staphylococcus equorum</i> | 100% |
| | Q [r] | <i>Staphylococcus equorum</i> | 100% |
| DP4 ⁻³ | H | <i>Sporosarcina sp.</i> | 100% |
| | H [r] | <i>Sporosarcina sp.</i> | 100% |
| DP5 ⁻² | J | <i>Thalassospira sp</i> | 99% |
| | J [r] | <i>Thalassospira sp</i> | 99% |
| DP5 ⁻³ | C | <i>Micrococcus luteus</i> | 99% |
| | C [r] | <i>Micrococcus luteus</i> | 100% |
| CONT1 ⁻² | P | <i>Actinobacterium</i> | 100% |
| | P [r] | <i>Actinobacterium</i> | 100% |

[r]: reverse sequence.

Table 2.3 shows the identification of isolates by sequencing of their 16S rDNA genes. The process was carried out for forward and reverse reactions using different primers. The probability values were between 99-100% showing a positive match and 95-98% showing a probable match.

2.4.4 Preparation of a Calibration Curve for BDHAC Analysis.

Prior to the measurement of residual amount of BDHAC in the growth medium after biodegradation experiment, a calibration curve was prepared with 20,10,5 and 2mg/l BDHAC standards following the procedures described in section 2.3.7 and absorbance values from the spectrophotometer obtained for these concentration were recorded.

Table 2.4: Relationship between BDHAC Concentration and Absorbance

| Concentration of Std (mg/l) | Absorbance at 450 (nm) |
|-----------------------------|------------------------|
| 20 | 2.861 |
| 10 | 1.823 |
| 5 | 0.925 |
| 2 | 0.308 |

Concentration of calibration standards are shown in Table 2.4. A calibration curve (Figure 2.3) was obtained by plotting the absorbance values against the concentration of calibration standards. The figure showed linearity ($R^2 = 0.978$) and a low detection limit of the instrument. The equation of the line generated was used to measure BDHAC concentrations, which in turn were used to work out the degradation values.

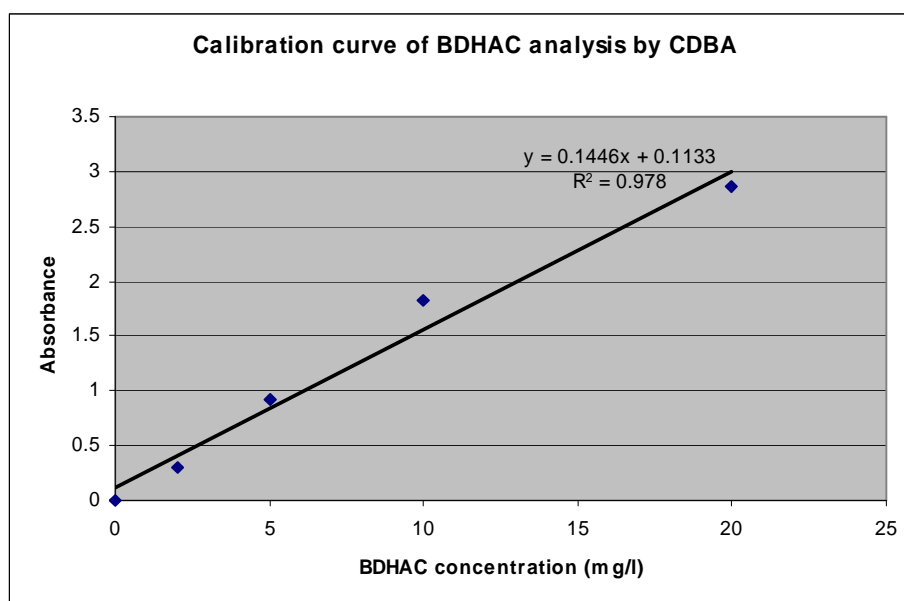


Figure 2.3 Calibration Curve for Colorimetric Dye Binding Assay (CDBA)

The equation of the line computed from the calibration curve is given as:

$$y = 0.1446x + 0.1133$$

Where y is the absorbance and x is the concentration (mg/l or $\mu\text{g/ml}$) of BDHAC.

2.4.5 BDHAC Recovery Analysis

Quaternary ammonium compounds, being cationic surfactants, adsorb to the surface of materials (Swisher, 1970); therefore it is important to differentiate between losses of these compounds due to adsorption and biodegradation. For this reason, a recovery experiment was carried out following the same procedure as the actual experiment but without incubation for a specified period of time with bacterial strains. The results obtained from the measurement were subsequently used to correct the percent degradation values obtained from the degradation experiments so as to ascertain the fact that of the removal of BDHAC from the incubation mixture was due to biodegradation. Percent recovery calculations are shown in Appendix A.1.

Table 2.5 Recovery of BDHAC from Sample Solutions

| Volume of Stock std added (μ l) | Volume of std. + medium (ml) | Amount of BDHAC added (mg) | Mean Absorbance at 450 (nm) | % Recoveries | | | |
|--------------------------------------|------------------------------|----------------------------|-----------------------------|--------------|------|-------|------------------|
| | | | | 1 | 2 | 3 | Mean |
| 100 | 100.1 | 5 | 1.639 \pm 0.32 | 40.88 | 34.1 | 51.64 | 42.21 \pm 8.84 |

Error margin = ± 1 Standard Deviation (n = 3 extractions)

Table 2.5 shows mean absorbance value and percent recovery value for 5mg BDHAC. A recovery of 42.21% was recorded for the test substance (see Appendix A.1) for a sample calculation.

2.4.6 Measurement of BDHAC using the CDBA

It was important to check the amount of BDHAC after enrichment culture experiment. This section shows the results obtained from quantification experiments that were carried out.

Quantification of BDHAC by Colorimetric Dye Binding Analysis: After the isolation of bacteria that may have degraded BDHAC that was contained in the enrichment media in the flasks, it was important to check for possible utilization of this substance by the isolates. Therefore the amount of BDHAC in the flasks after the enrichment experiment was quantified using Colorimetric Dye Binding Analysis (CDBA). Results for CDBA are summarised in Table 2.6. Calculations showing the determined amount of BDHAC in relation to amount adsorbed can be seen in Appendix B.1

Table 2.6 Quantification of BDHAC in the Test and Control Samples by CDBA

| Incubation Time (Days) | Samples | Absorbance | Amount of BDHAC (mg) | % Degradation |
|---------------------------|---------|------------|----------------------|---------------|
| 43 | DP1 | 0.878 | 0.627 | 49.81 |
| | DP2 | 0.629 | 0.423 | 66.15 |
| | DP3 | 0.554 | 0.360 | 71.19 |
| | DP4 | 0.997 | 0.722 | 42.22 |
| | DP5 | 0.748 | 0.518 | 58.53 |
| | Control | 1.340 | 0.995 | 20.40 |

The degradation values were corrected for adsorption with the calculated mean percent recovery value of 42.21%. The highest percent degradation value (71.19) from CDBA was recorded for sample DP3 (Table 2.6) after allowance was made for adsorption onto the flask. The correction for adsorption was achieved by using the value obtained from the recovery to compute the actual losses due to biodegradation. Also high percent degradation values (66.15 and 58.53) were recorded for samples DP2 and DP5 respectively. Percent degradation values that were recorded for samples DP1 and DP4 were close (49.81 and 42.22) respectively and the least percent degradation value (20.40) was observed for the control sample. The result obtained for the control sample suggested that the isolate present in the flask managed to degrade BDHAC to a small extent due to inactivity of the 1% mercuric chloride that was initially added to stop bacterial growth. This result confirmed a higher extent of degradation in the test samples than in the control.

2.4.7 Preparation of Calibration Curve for the Quantification of BDHAC by ESI-MS/MS.

A calibration curve was prepared for the quantification of BDHAC by ESI-MS/MS. This was also used to check the sensitivity of the Tandem Mass Spectrometer. This process was

carried out prior to the quantification of residual BDHAC by Electrospray Ionisation Mass Spectrometric Analysis. 10 μ l of the calibration standards prepared (section 2.3.7) were injected into the mass spectrometer in the positive ion mode and peaks showing amounts of BDHAC and the internal standard were obtained. A ratio of BDHAC peak areas to that of C14 internal standard was computed and the results were plotted against the concentration of calibration standards (Figure 2.4). The calibration curve showed linearity ($R^2=0.902$) over the concentration range of 0.123-126 μ g/ml with a low detection limit. The equation of the straight line generated from the calibration curve was used to measure BDHAC concentrations, which in turn were used to work out the degradation values.

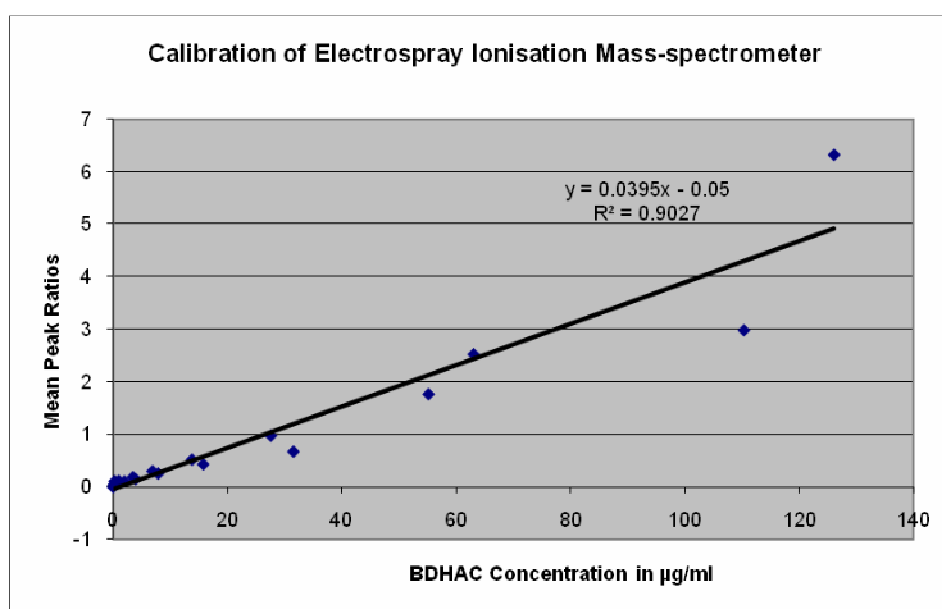


Figure 2.4 Calibration Curve for BDHAC Analysis by ESI-MS/MS

The equation of the line deduced from the calibration curve is given as

$$y = 0.039x - 0.05$$

where y is the mean peak area ratio of the test substance to the internal standard and x is the unknown concentration (μ g/ml) of the test substance (BDHAC).

2.4.8 Quantification of BDHAC by Electrospray Ionization Mass-Spectrometry

Electrospray ionisation tandem mass spectrometry has been shown to be the method of choice for the analysis of oilfield specialty chemicals owing to its specificity and sensitivity, often with very low detection limits (Grigson et al., 2000). The technique allows direct ionisation from solution phase and can perform high molecular weight determination through the use of multiply charged ions (Raffaelli and Bruins, 1991)

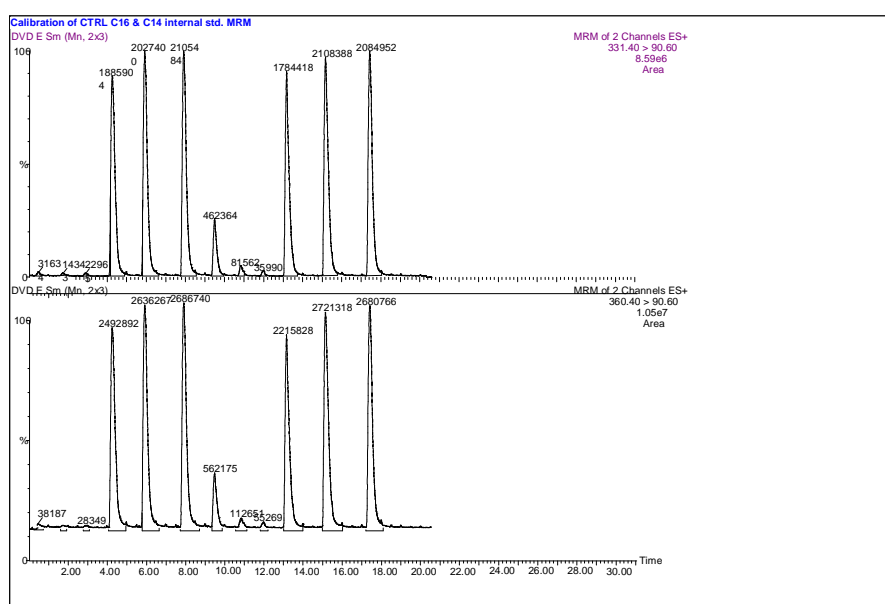


Figure 2.5 Chromatogram for the Quantification of BDHAC in Control Sample

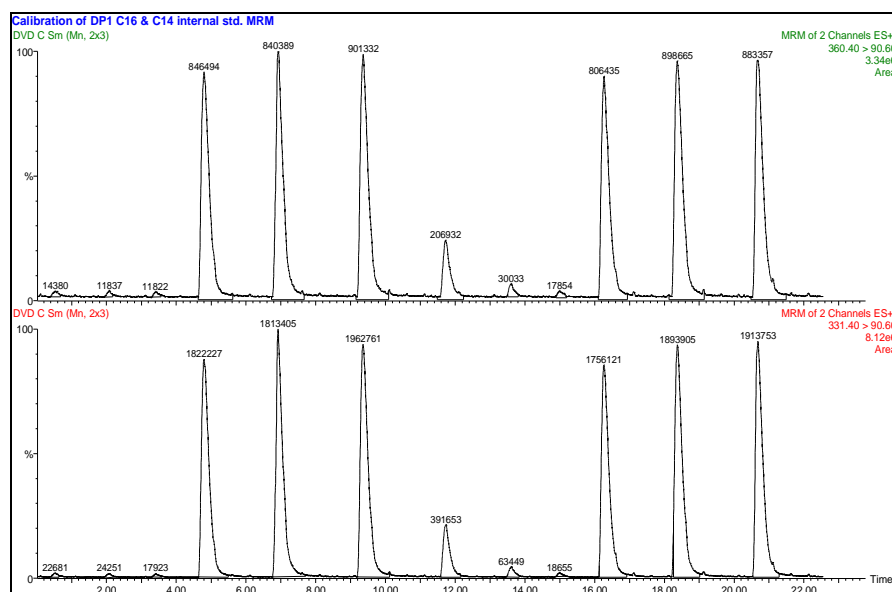


Figure 2.6 Chromatogram for the Quantification of BDHAC in Sample DP1

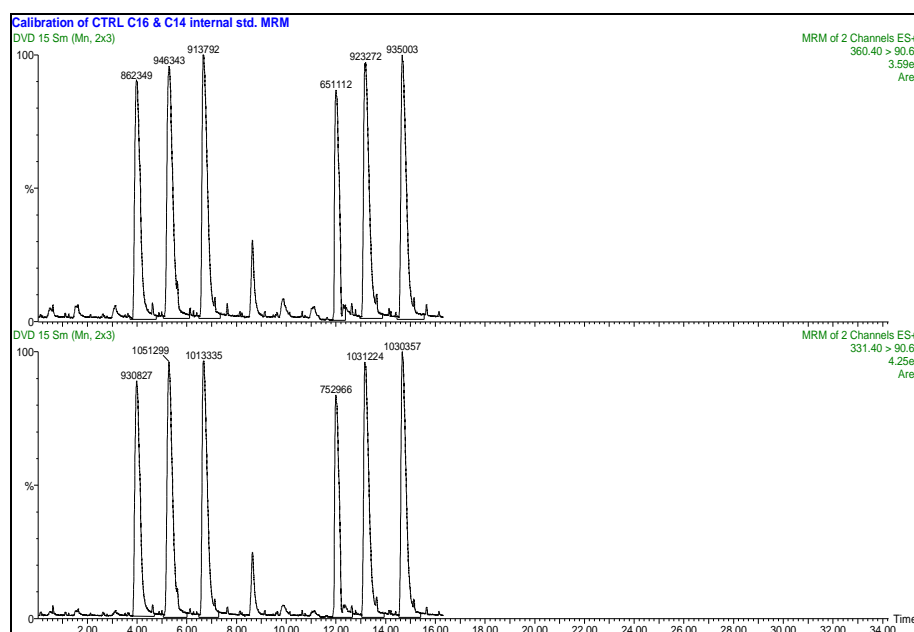


Figure 2.7 Chromatogram for the Quantification of BDHAC in sample DP2

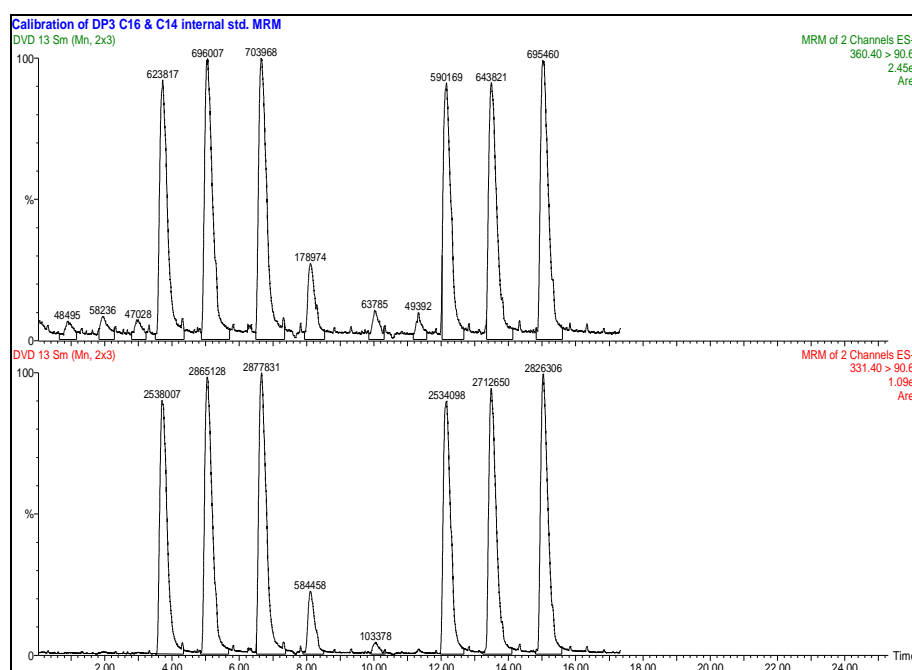


Figure 2.8 Chromatogram for the Quantification of BDHAC in sample DP3

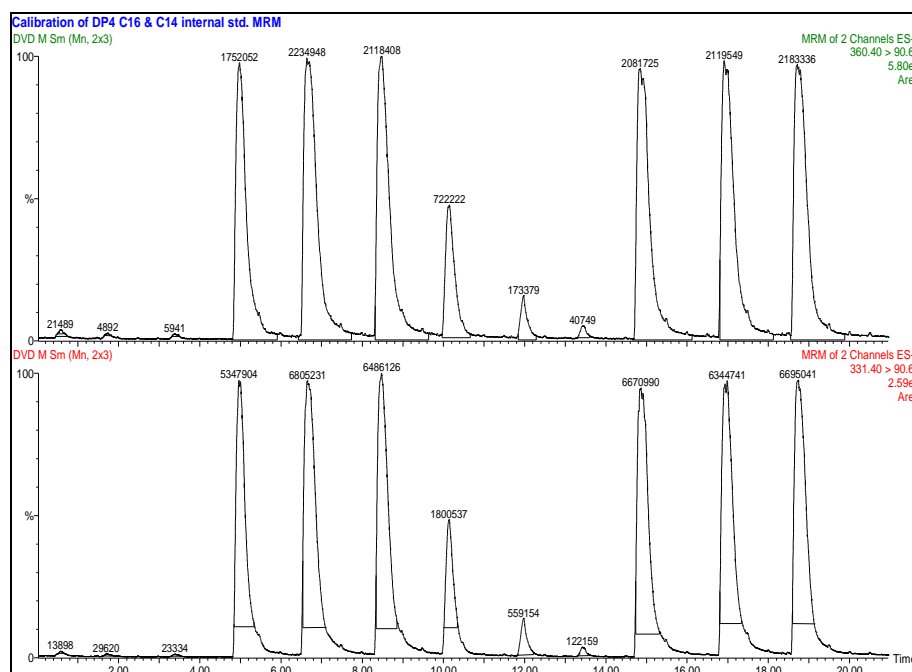


Figure 2.9 Chromatogram for the Quantification of BDHAC in sample DP4

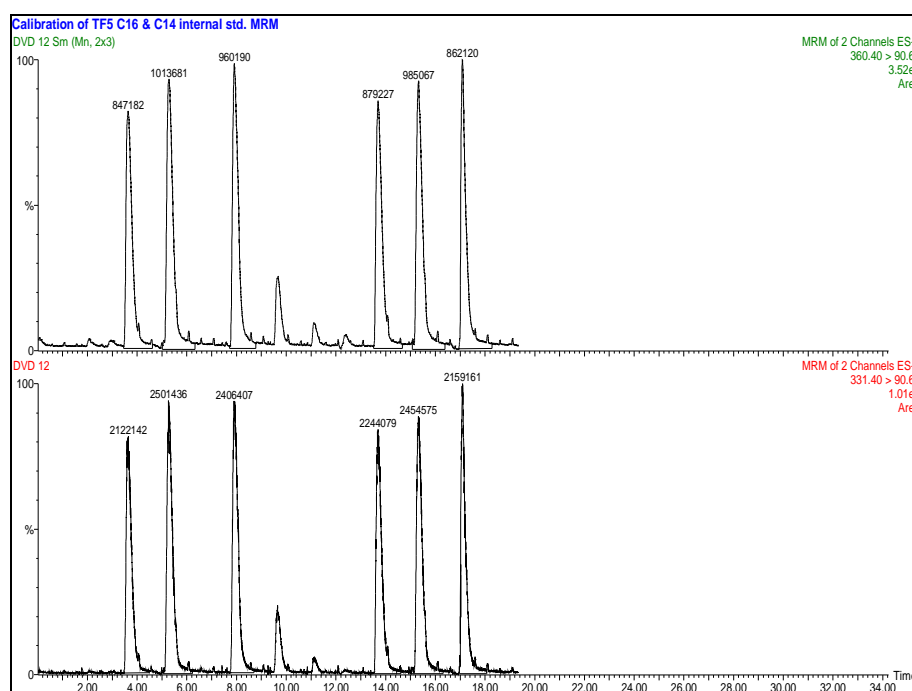


Figure 2.10 Chromatogram for the Quantification of BDHAC in sample DP5

Table 2.7 Quantification of BDHAC in the Test and Control Samples by ESI-MS/MS

| Incubation Time (Days) | Sample | BDHAC Peak Areas | Pk Areas of IS | Peak Ratios (A/B) | Amount of BDHAC (mg) | % Deg |
|---------------------------|---------|------------------|----------------|-------------------|----------------------|-------|
| | | (A) | (B) | | | |
| 43 | DP1 | 862779 | 1860362 | 0.464 | 0.325 | 73.99 |
| | DP2 | 871979 | 968335 | 0.900 | 0.617 | 50.62 |
| | DP3 | 658874 | 2725670 | 0.242 | 0.185 | 85.23 |
| | DP4 | 2081670 | 6391672 | 0.326 | 0.238 | 80.98 |
| | DP5 | 924578 | 2314633 | 0.399 | 0.577 | 53.88 |
| | Control | 2572302 | 1999424 | 1.287 | 0.846 | 32.33 |

Figures 2.5-2.10 show the positive ion ES-MS chromatograms for C16/C14 Quaternary ammonium compounds. The C16 Quat (BDHAC) is the test substance while the C14 Quat is the internal standard. The y axis is the % abundance of the analyte and the x axis is the retention time in minutes. The peaks shown at the top in the figures at transition of 360.40→90.60 represent replicate injections of the test substance (10µl BDHAC) (first three peaks) followed by replicate injections of blank samples (90:10 methanol/water) to check for carry over of BDHAC. The peaks below at transition of 331.40→90.60 represent C14 Quat internal standard that was added to the test samples at the start of the analysis. The concentration of the internal standard added to test samples in the quantification experiment was the same as performed for the calibration standards (section 2.3.7).

The peaks shown on the figures were quantified and the mean areas of the peaks for the analyte and the internal standard were used to compute the percent degradation values (see Appendix B.2). The highest percent degradation value of 85.23 was recorded for sample DP3 followed by sample DP4 with the value of 80.98% (Table 2.7). Also, a high percent degradation (73.99%) value was observed for sample DP1. Values recorded for DP2 and DP5 were close (50.62 and 53.88) % respectively. The lowest percent degradation value (32.33%) was recorded for the control sample. The low percent degradation value observed for the control sample confirmed that there was difference between the amount of residual BDHAC in the control and test samples.

Results for the quantification of BDHAC by CDBA and ESI-MS (Tables 2.6 and 2.7) revealed that BDHAC added to the growth medium at the start of the experiment was degraded by microorganisms present in the growth medium.

The residual concentrations of BDHAC measured following incubation with the bacterial strains differed for the two different analytical methods. The Colorimetric Dye Binding method is an assay for quaternary ammonium salts in general whereas ESI-MS/MS measured BDHAC specifically. As such, ESI-MS/MS would potentially be the method of choice for these determinations. Unfortunately, due to ongoing problems with the tandem mass spectrometer it was not possible to investigate more fully differences in the analytical data. However, both methods showed degradation of BDHAC compared to the control. The dye binding assay was subsequently used as the main analytical tool in this study, being a recognised method for determining the compounds under investigation.

2.5 Discussion

Micro-organisms were isolated from marine sediments in a growth medium in the presence of 0.05 mg/ml benzyldimethyl-hexadecylammonium chloride (BDHAC) during 43 days incubation. The isolates were identified by phenotypic characterisation tests and partial sequencing of their 16S-rDNA genes as *Bacillus subtilis*, *Micrococcus luteus* E, *Bacillus niabensis*, *Thalassospira* sp., *Rhodospirillaceae*, *Staphylococcus equorum*, *Sporosarcina* sp., *Thalassospira* sp, *Micrococcus luteus* C and *Actinobacterium*. Nishiyama and Nishihara (2002) reported the isolation two strains of bacteria F7 and F2 from activated sludge of a waste water treatment plant. They subsequently identified the strains as *Pseudomonas fluorescens*. The strains identified in this study were of several genera different to *Pseudomonads*.

Results obtained revealed that *Bacillus subtilis* was a representative of the microorganisms that were present in sample DP1. The bacterium was identified as an oxidase and catalase positive, gram positive motile rod. Also, *Micrococcus luteus* E and *Bacillus nianensis* were identified in samples from sample DP2 at the end of the enrichment experiment. The strains were oxidase and catalase positive, Gram-positive motile rod and coccus (Table 2.2). *Thalassospira* sp. and *Rhodospirillaceae* were identified from sample DP3 and these strains were shown to be oxidase and catalase positive, Gram-negative motile rods. In addition, *Staphylococcus equorum*, *Sporosarcina* sp isolated from sample DP4 were catalase and oxidase positive, Gram-positive and negative coccus and rods respectively (Table 2.2). *Thalassospira* sp. and *Micrococcus luteus* C were representatives of the strains present in sample DP5 and were identified as catalase and oxidase positive, Gram-negative and positive rod and coccus respectively. From the control sample, a bacterium identified as *Actinobacteria* was isolated, even though 1% mercuric chloride (section 2.3.3) was added to the flasks before incubation. Although bacteria isolated in the control sample was not of any particular interest, it was noted that it was less able to degrade BDHAC compared to other isolates.

Generally, it was observed that the isolated strains of bacteria were apparently able to degrade BDHAC during 43 days of incubation. The results confirm the suggestion by Larson and Vashon, (1983) that QACs are ultimately biodegradable at low concentrations

in river surface water and/or sediments. Also, deductions made from the results obtained from the isolation and quantification experiments support the fact that natural communities of micro-organisms in various habitats have an amazing physiological versatility and are able to metabolise and often mineralise a number of organic molecules (Alexander 1973; Hanson, 1997).

Residual amounts of BDHAC after 43days incubation experiment were quantified by CDBA and ESI-MS analysis. The key to the success of the ESI-MS technique for the quantification of residual BDHAC from the degradation studies lies in the softness of the electrospray ionisation process and the fact that the fast flow approach utilised ensures short sample throughput and comparatively short analysis times (Klug and Fresenius 1987 ; Nishikawa et al., 1994 and 1996). Results obtained from CDBA and ESI-MS analysis which started from the same samples did not agree quantitatively but revealed high percent degradation values for samples DP3 (71.19 and 85.23), and close degradation values for DP5 (58.53 and 53.88) % respectively. In addition, results showed low percent degradation values (20.40 and 32.33) respectively for the control sample. Based on the differences observed in the results obtained for test samples and the control, the reliability of the techniques may be questioned. However, Waters and Kupfer, (1976) had successfully analysed hexadecyltrimethylammonium chloride using CDBA and Grigson et al., (2000) reported that the techniques are very reliable in the analysis of oilfield specialty chemicals. The differences in the results observed may have been because correction for adsorption was not carried out on the results of the ESI-MS analysis. The two techniques were involved in this study because of the frequent breakdown of the mass spectrometer that hindered smooth operation of the ESI-MS technique. For this reason, CDBA would be used mostly for sample analysis in the course of this study.

Generally , a variety of organisms were isolated in varying amounts in different samples and this shows that a range of organisms is able to survive and perhaps degrade BDHAC under enrichment conditions.

2.6 Conclusion

Data obtained from this chapter strongly support the position that BDHAC was biodegradable in the growth medium. A more detailed investigation involving higher concentrations of BDHAC was carried out in Chapter 3 to confirm this conclusion.

CHAPTER 3

SECTION A

BIODEGRADATION OF BDHAC BY EIGHT STRAINS OF BACTERIA SELECTED FROM PRELIMINARY BIODEGRADATION STUDIES.

In this section further investigation into the degradation of BDHAC by eight strains of bacteria is described.

3.1 Introduction

Initial investigations in Chapter 2 supported the hypothesis that BDHAC was biodegradable in the growth medium. In this chapter, more detailed studies on the biodegradation of BDHAC by selected strains of bacteria are described. The method involved was as reported by Takenaka et al., (2007).

3.2 Aim of the Study

The aim of the study was to investigate the extent of degradation of different amounts of BDHAC by 8 strains of bacteria selected from the 10 strains of bacteria isolated, identified and preserved at -70⁰C in a freezer. The 8 strains were those that were isolated from test samples. It was important to examine how these strains of bacteria would grow in the presence of different amounts of BDHAC as a source of carbon and energy. Yeast extract was used as an additional nutrient in these experiments. Therefore the extent of growth and degradation of BDHAC was being investigated in an optimum growth medium. The approach was different from that reported in Chapter 2 because the period of incubation was reduced to 28 days and the amount of BDHAC used for the degradation experiments was

increased. The study will give information on the concentrations of BDHAC that support the growth of the strains and the ability of each strain to utilise this substance. The procedures that were involved in carrying out the study are described in the sections below.

3.3 Materials and Methods

Benzyltrimethylhexadecylammonium chloride (BDHAC) (Sigma), potassium phosphate (K_2HPO_4), sodium chloride (NaCl), yeast extract, magnesium sulphate ($MgSO_4 \cdot 7H_2O$), calcium chloride ($CaCl_2 \cdot 2H_2O$), copper (II) sulphate ($CuSO_4 \cdot 5H_2O$), zinc chloride ($ZnCl_2$), iron (II) sulphate ($FeSO_4 \cdot 7H_2O$) and ammonium nitrate (NH_4NO_3) were purchased from Fisher Scientific Company, UK. The reagents were of analytical grade.

3.3.1 Preparation of Growth Media

Preparation of media was performed according to the method of Takenaka et al., (2007). Two solutions were prepared separately and later mixed to produce an enrichment medium for the bacteria. The initial separation was essential to avoid problems when autoclaving.

Medium A: Contained 0.08 g of KH_2PO_4 , 4.16 g of K_2HPO_4 , 0.4 g of NaCl, 0.16 g of yeast extract, 0.40 g of NH_4NO_3 , and deionised water in a total volume of 680 ml, with the final pH adjusted to 7.0 with 6N HCl.

Medium B: Contained 4 g of $MgSO_4 \cdot 7H_2O$, 4 mg of $CaCl_2 \cdot 2H_2O$, 4 mg of $CuSO_4 \cdot 5H_2O$, 4 mg of $ZnCl_2$, and 4 mg $FeSO_4 \cdot 7H_2O$, and deionised water to total volume of 120 ml.

3.3.2 BDHAC Degradation Experiment

7 ml of Medium A was pipetted into 8 test tubes and 3 ml of Medium B was pipetted into another set of 8 test tubes and all were autoclaved at $121^{\circ}C$ for 15 minutes. After autoclaving, medium B was poured into medium A bringing the total volume of medium (A+B) in the test tubes to 10 ml. This was performed for eight batches of 8 test tubes bringing the total number of test tubes to 64.

To the 64 test tubes above, 0.8, 0.6, 0.5, 0.4, 0.2, 0.1, 0.01 ml of 5g/100ml BDHAC standard (**Std 1**) was added in batches of 8 to give concentrations of 3.70, 2.83, 2.38, 1.98, 0.98, 0.50, 0.05 mg/ml. In these batches of 8 were control samples which no BDHAC was added. To each batch of tubes a different bacterium isolated from the previous enrichments: *Micrococcus luteus* E and C, *Bacillus subtilis*, *Bacillus niabensis*, *Sporosarcina sp.*, *Thalassospira sp*, *Rhodospirillaceae* and *Staphylococcus equorum* respectively (see section 2.4.3) was introduced.

The test tubes with no Quat added served as the control for the experiment. All inoculated test tubes were incubated at 25⁰C for 28 days. During the incubation period, 3.2 ml samples were withdrawn for optical density (OD) determination and the quantification of the amount of residual BDHAC by Colorimetric Dye Binding Assay. These measurements were carried out as described below.

3.3.3 Determination of Optical Density

Optical density (OD) is used as a measure of the concentration of bacteria in a suspension. During OD measurement, visible light passes through a cell suspension and the light is scattered. Greater scattering indicates more turbidity of the cell suspension. The amount of light scattered can be measured in a spectrophotometer as the absorbance.

OD of the cultures was determined using Ultraspec 1000E Spectrophotometer manufactured by Pharmacia Biotech, Cambridge. England. OD measurements were at a wavelength of 650 nm. 800 µl of blank (sterile, uninoculated media) and sample solution were pipetted into disposable polystyrene cuvettes using sterile technique. The cuvettes were labelled on the frosted portion at the top.

The cuvette with the blank was placed in the holder on the spectrophotometer and on the home tab on the visual display, a fixed wavelength was selected. On the next screen, a check was carried out to ensure that the fixed wavelength selected was 650 nm. In the lower left of the screen, was a display that read “vis on” indicating that the instrument was ready for use. A click sound was also heard which indicated that the spectrophotometer was at a baseline absorbance of the blank. The cuvette with the blank was then replaced with that with the sample solution and OD was measured.

3.3.4 Quantification of the Amount of Residual BDHAC by Colorimetric Dye Binding Assay

Quantification of residual BDHAC was performed by CDBA as was previously described in Section 2.3.7. Details of the method are described in Steps 1 and 2 below

Step1

On day 28 of incubation, the volumes remaining in the test tubes were 7.6, 7.4, 7.3, 7.2, 7.0, 6.9, 6.81 and 6.8ml respectively for the different concentrations of BDHAC, after 3.2 ml had been taken out for optical density determination. The remaining sample was then centrifuged to obtain a clear solution for CDBA. The supernatants obtained after centrifugation, were extracted with 10 ml of dichloromethane (DCM) in a separating funnel and the resulting DCM extracts (10 ml) were collected in scintillation vials. 1 ml of the 10 ml DCM extract was transferred with a pipette to a 50 ml volumetric flask and blown to dryness under a gentle stream of nitrogen. The flasks were then filled to the mark with seawater which now contained the residual BDHAC.

Step2

The contents of the flasks were analysed using the procedures described in section 2.3.7.

Preparation of Calibration curve: A calibration curve was prepared as described in section 2.3.7.

3.3.5 BDHAC Recovery Analysis

Quaternary ammonium compounds have been shown to adsorb to glassware and this makes it difficult to distinguish removal due to adsorption from that due to biodegradation (Swisher, 1970). For this reason, a recovery experiment was performed to correct losses due to adsorption of BDHAC to glassware. 10 ml test tubes were filled with media and different volumes i.e. 0.8, 0.6, 0.5, 0.4, 0.2, 0.1 and 0.01 ml of 5g/100ml BDHAC standard (Std 1. Section 3.3.2) were added to these tubes to give the resulting volumes of 10.8, 10.6, 10.5,

10.4, 10.2, 10.1 and 10.01 ml respectively. No BDHAC was added to the control test tubes and the volume of media was 10 ml. After the addition of the stock standards the test tubes were allowed to sit for 24 hrs before 3.2 ml (same amount that was removed from the flasks for optical density measurement during the degradation experiment) was removed. The new volumes in the test tubes were 7.6, 7.4, 7.3, 7.2, 7.0, 6.9, 6.81 ml respectively and 6.8 ml in the control. These volumes were extracted with 10 ml of DCM in a 250 ml separating funnel and 1 ml of the 10 ml DCM extract was transferred via a pipette to a 50 ml volumetric flask. The DCM extract was blown to dryness in a gentle stream of N₂ and the residues in the flasks were taken up in seawater to the 50 ml mark. The 50 ml solutions in the flasks were analysed for the concentration of residual BDHAC in solution by Dye Binding Assay. The experiment was carried out in duplicate.

3.4 Results

3.4.1 Optical Density Values Showing Growth of Bacteria at Different Concentrations of BDHAC.

Results from optical density measurements at different concentrations of BDHAC for the different strains of bacteria are presented in this section.

Micrococcus luteus E

Figures 3.1 and 3.2 show optical density (OD) values for *Micrococcus luteus* E in the growth medium at different concentrations of BDHAC following 28 days of incubation. Growth levels increased in the first 14 days of incubation at BDHAC concentrations of 0.5, 1.92 and 2.83mg/ml only to decrease as the experiment progressed to day 21.

BIODEGRADATION OF BDHAC BY EIGHT STRAINS

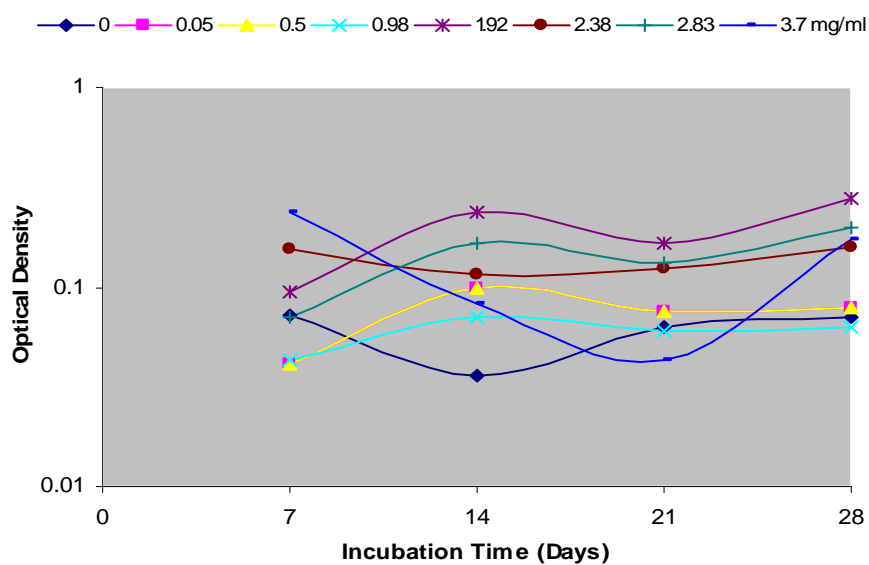


Figure 3.1 Growth of *Micrococcus luteus* E at Different BDHAC Concentrations.

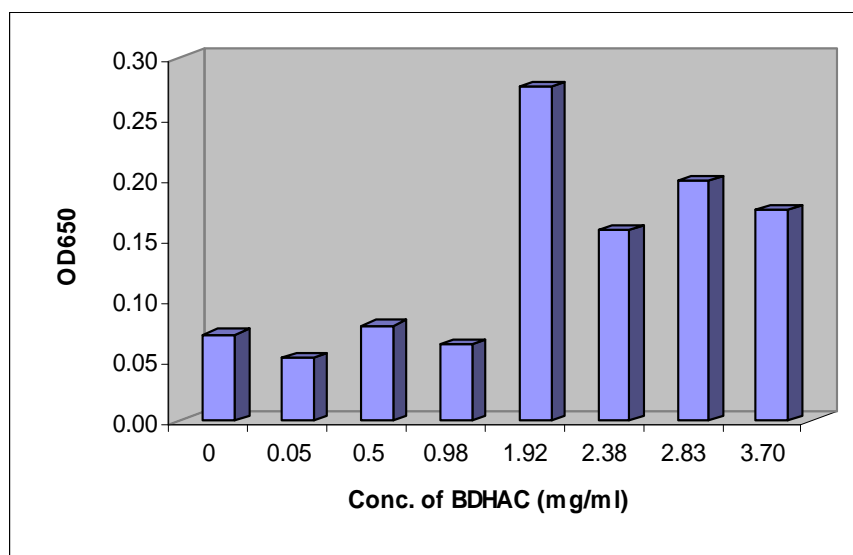


Figure 3.2 Optical Density as a Function of BDHAC Concentration for *Micrococcus luteus* E. on Day 28 of Incubation.

By day 28 of incubation growth levels increased again at concentrations of 1.92, 2.38, 2.83 and 3.70 mg/ml when compared to the control that lacked BDHAC. The decrease in growth observed by day 21 of incubation may have been due to an inadequate production of new cells by this strain of bacteria in the growth medium. It was surprising to observe that growth levels of the strain in the control were almost the same as in media containing 0.05, 0.5 and 0.98 mg/ml BDHAC. Growth results for this strain generally showed that up to 3.70 mg/ml BDHAC supported its growth when the Quat was present in the growth medium as a carbon and energy source.

Bacillus niabensis

Results for the growth of *Bacillus niabensis* are shown in Figures 3.3 and 3.4. Growth of the strain was fairly minimal at the different BDHAC concentrations throughout the duration of the experiment when compared to the control. The first 14 days of incubation showed a slight increase in growth levels at BDHAC concentrations of 0.05, 0.5, 0.98 and 1.92 mg/ml.

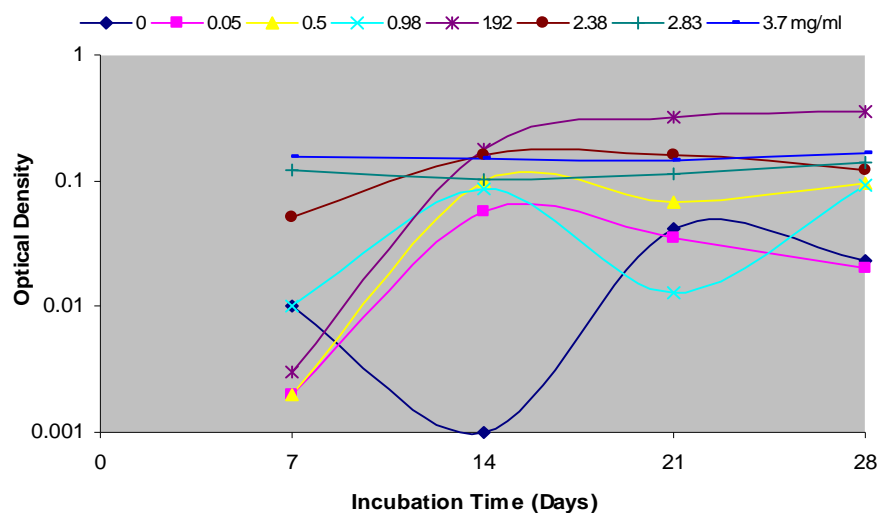


Figure 3.3 Growth of *Bacillus niabensis* at Different BDHAC Concentrations.

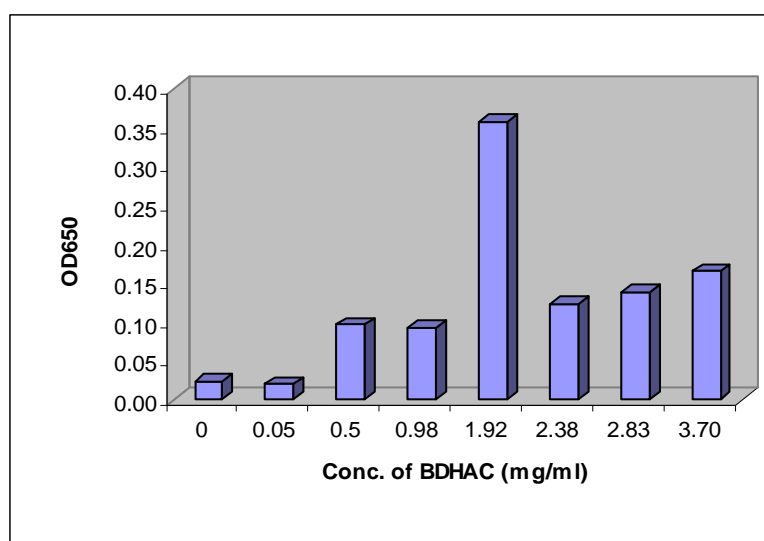


Figure 3.4 Optical Density as a Function of BDHAC Concentration for *Bacillus niabensis* on Day 28 of Incubation.

By day 21 of the incubation, growth levels were shown to decrease. Growth of the strain remained at the same level throughout the incubation period at concentrations of 2.83 and 3.70 mg/ml BDHAC. On the 28th day of incubation, growth of the strain increased at concentrations of 1.92, 2.38, 2.83 and 3.70 mg/ml when compared to the control. The highest level of growth was recorded for 1.92 mg/ml BDHAC while the lowest was recorded for 0.05mg/ml (Figure 3.4). These results suggested that *Bacillus niabensis* G was able to grow in medium containing different concentrations of BDHAC and that up to 3.70 mg/ml of the Quat did not inhibit the growth of the bacteria.

Bacillus subtilis

Results for the growth of *Bacillus subtilis* are shown in Figures 3.5 and 3.6. From the results obtained, it appeared the bacterium went into a lag phase during incubation. There was slow growth of this strain within the first 21 days of incubation at 2.83 and 3.70 mg/ml

BDHAC and a slight increase in growth was observed at 0.05, 0.5, 1.92 and 2.38 mg/ml BDHAC (Figure 3.6).

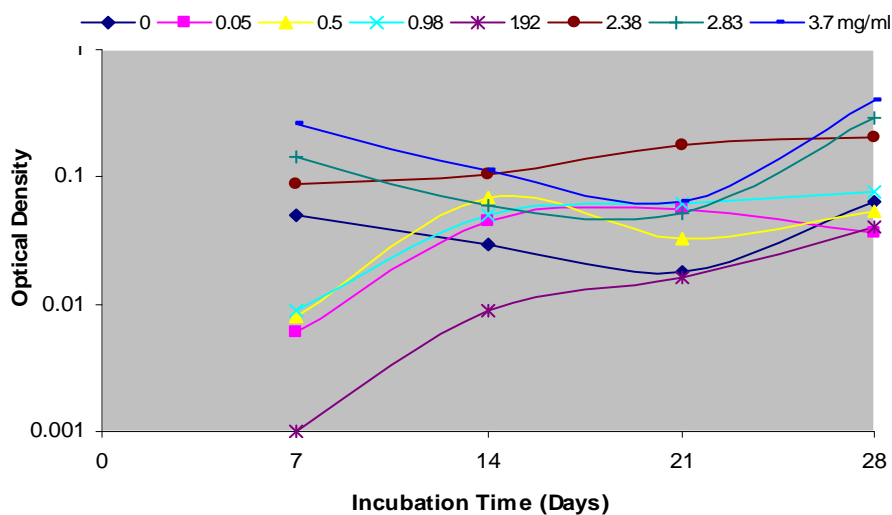


Figure 3.5 Growth of *Bacillus subtilis* F at Different BDHAC Concentrations.

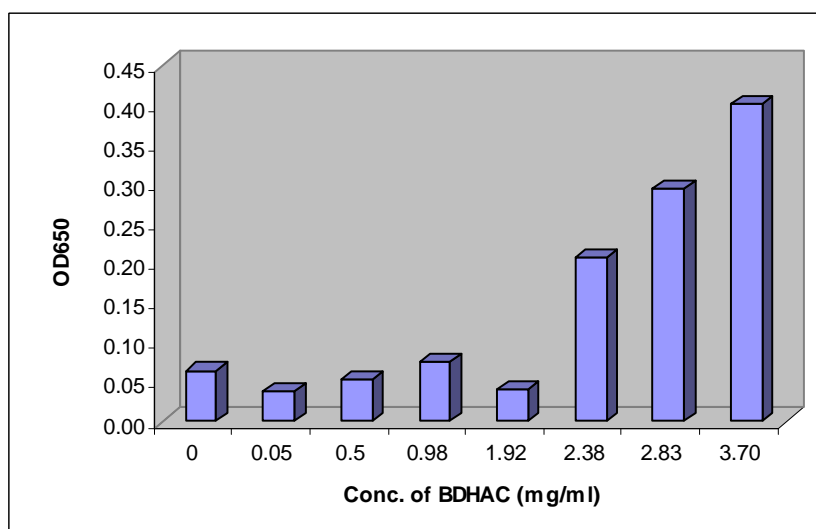


Figure 3.6 Optical Density as a Function of BDHAC Concentration for *Bacillus subtilis* on Day 28 of Incubation.

On day 28 of incubation, growth of the bacterium increased at BDHAC concentrations of 2.38, 2.83 and 3.70 mg/ml when compared to the control. The highest growth level was recorded for 3.70 mg/ml while the lowest was observed at 0.05 and 1.92 mg/ml (Figure 3.6). It was surprising to observe that growth of the strain was lower at 0.05, 0.5 and 1.92 mg/ml than in the control that was without Quat. Growth results for this strain generally showed that BDHAC at 0.05-3.70 mg/ml supported its growth when the Quat was present as an organic substrate in the growth medium during 28days of incubation.

Rhodospirillaceae

Growth results for *Rhodospirillaceae* are show in figures 3.7 and 3.8. Increase in growth of the strain was observed at BDHAC concentrations 0.5, 1.92 and 2.83 mg/ml by days 7 to 28 of incubation. Steady growth was observed at 2.38 and 3.70 mg/ml BDHAC in the duration of the experiment.

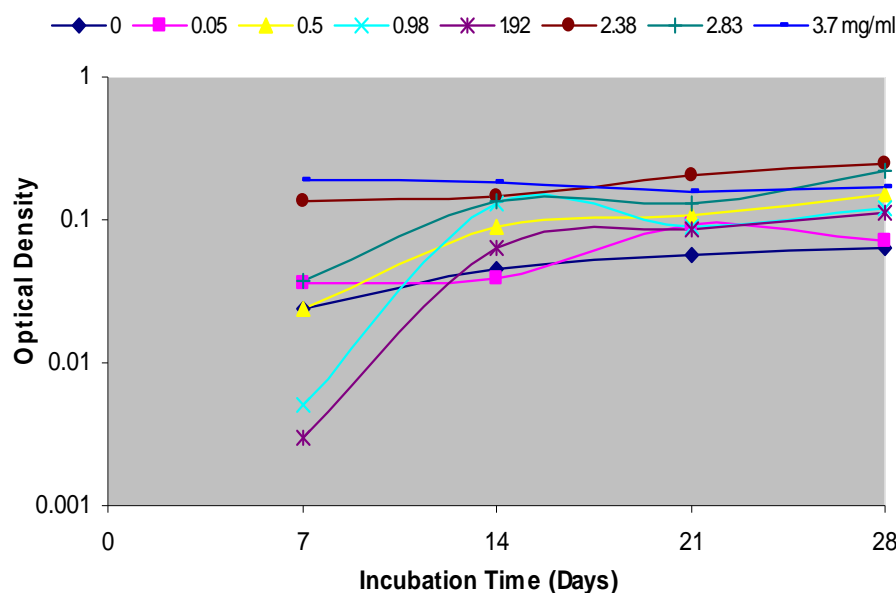


Figure 3.7 Growth of *Rhodospirillaceae* at Different BDHAC Concentrations.

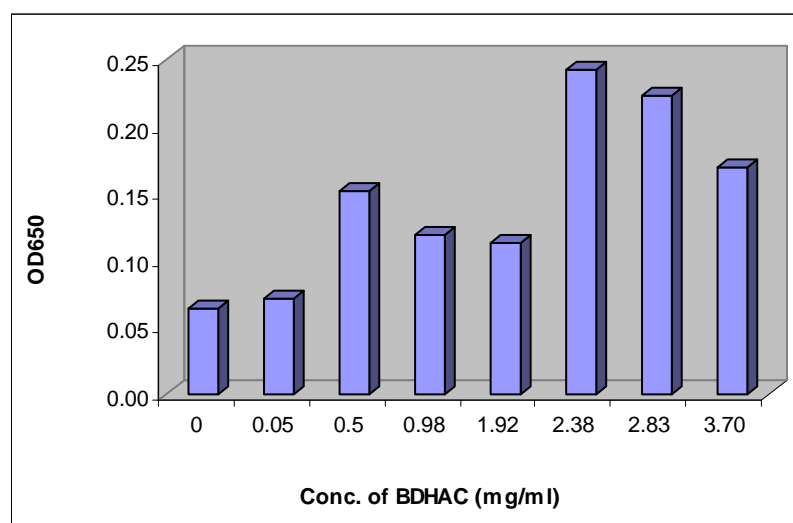


Figure 3.8 Optical Density as a Function of BDHAC Concentration for *Rhodospirillaceae* on Day 28 of Incubation.

On day 28 of incubation, increased growth levels were recorded at BDHAC concentrations of 0.5, 2.38, 2.83 and 3.70 mg/ml when compared to the control. The highest and lowest growth levels were observed at 2.38 and 0.05 mg/ml respectively (Figure 3.8).

Generally, it was deduced from the results that the strain survived in growth medium containing up to 3.70 mg/ml BDHAC and may have utilised the Quat as a source of carbon and energy to make new cells.

Thalassospira sp

Figures 3.9 and 3.10 illustrate the growth of *Thalassospira sp* in growth medium with different concentrations of BDHAC. Growth of the strain was generally slow during the incubation period. At concentrations of 0.05 and 1.92mg/ml BDHAC, growth of the strain slightly increased as the experiment progressed. (Figure 3.10).

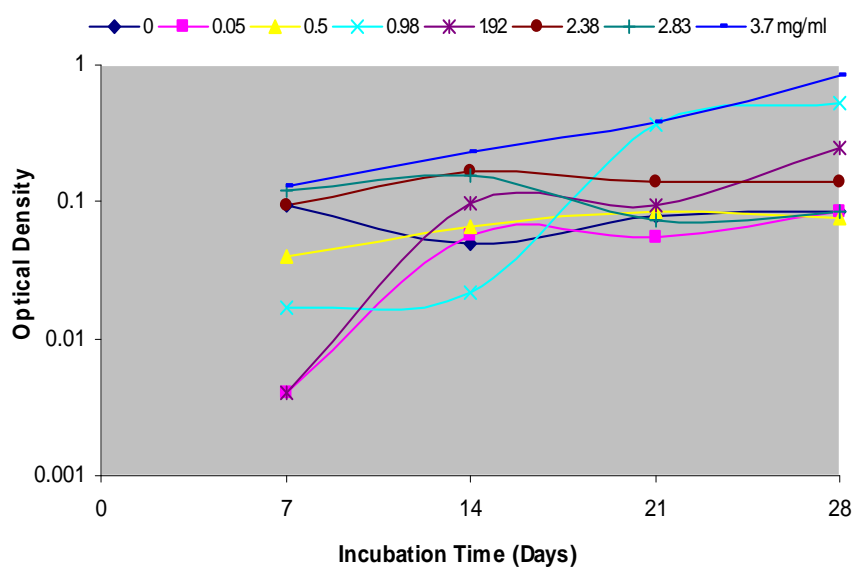


Figure 3.9 Growth of *Thalassospira sp.* at Different BDHAC Concentrations.

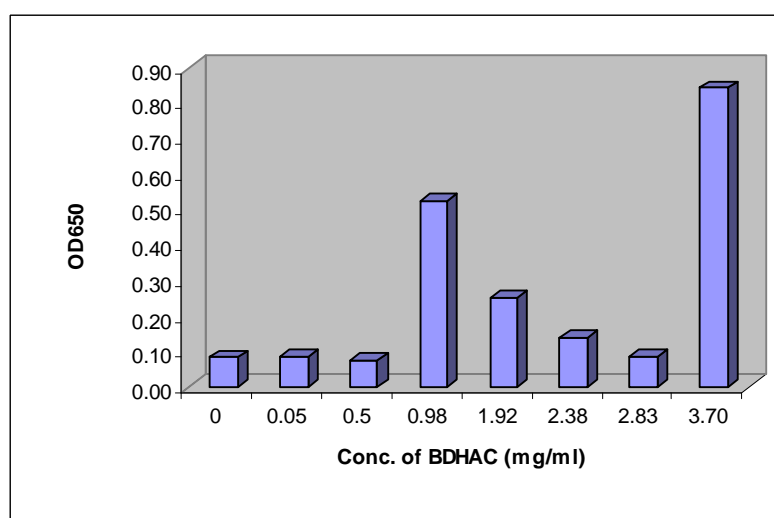


Figure 3.10 Optical Density as a Function of BDHAC Concentration for *Thalassospira sp.* on Day 28 of Incubation.

By the 28th day of incubation, increased growth levels were recorded for 0.98 and 3.70 mg/ml BDHAC compared to the control while limited growth was shown for BDHAC concentrations of 0.05, 0.5, 1.92 and 2.83 mg/ml (Figure 3.10). Growth results generally revealed that *Thalassospira sp.* was able to grow in medium containing as high as 3.70 mg/ml BDHAC during 28days of incubation and the high concentration of the Quat was not toxic to the bacterium.

Micrococcus luteus C

Growth results for this strain are shown in Figures 3.11 and 3.12. There was a general increase in growth of the strain by the first 14 days of incubation at different concentrations of BDHAC. Growth decreased at 0.5, 2.83 and 3.70 mg/ml by day 21 of incubation (Figure 3.12).

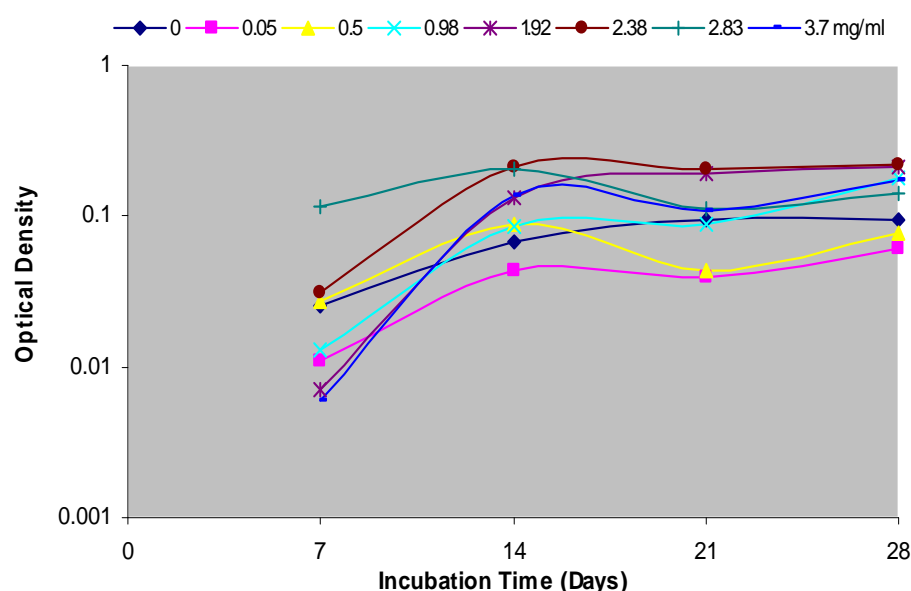


Figure 3.11 Growth of *Micrococcus luteus* C at Different BDHAC Concentrations.

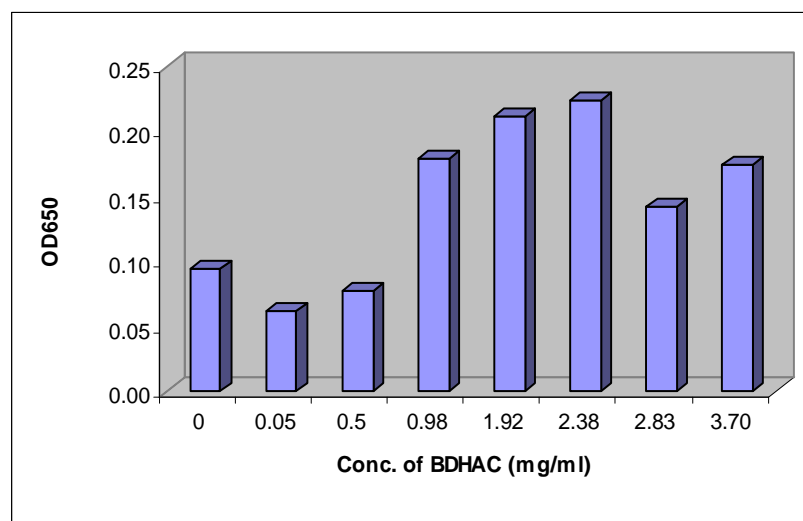


Figure 3.12 Optical Density as a Function of BDHAC Concentration for *Micrococcus luteus* C on Day 28 of Incubation.

Growth levels of the strain were shown to have increased at 0.98, 1.92, 2.38, 2.83 and 3.70 mg/ml BDHAC by day 28 of incubation when compared to the control. Limited growth were observed at 0.05 and 0.5mg/ml BDHAC compared to the control (Figure 3.12). The highest and lowest growth levels were recorded for BDHAC concentrations of 2.38 and 0.05 mg/ml respectively. Similar observation was made for the growth of *Rhodospirillaceae* (Figure 3.8). Generally, growth results revealed that 3.70 mg/ml of BDHAC present in the growth medium during a 28 day incubation period supported the growth of *Micrococcus luteus* C.

Staphylococcus equorum

Figures 3.13 and 3.14 show growth results for *Staphylococcus equorum*. Increased growth of the strain was observed at BDHAC concentration 0.05, 0.5 and 1.92 mg/ml during the period of incubation. Growth levels were steady at 2.83 and 3.70 mg/ml BDHAC while at 2.38 mg/ml, growth was shown to decrease by day 21 of incubation (Figure 3.14).

BIODEGRADATION OF BDHAC BY EIGHT STRAINS

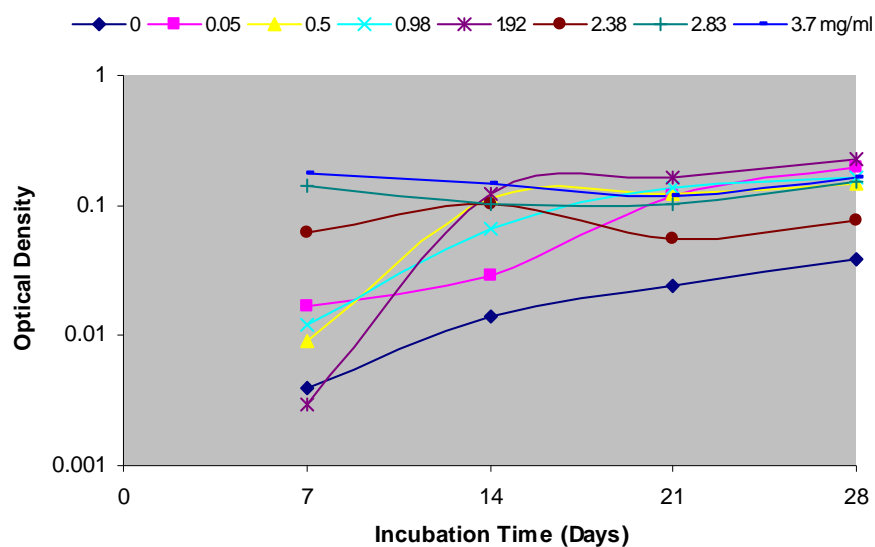


Figure 3.13 Growth of *Staphylococcus equorum* at Different BDHAC Concentrations.

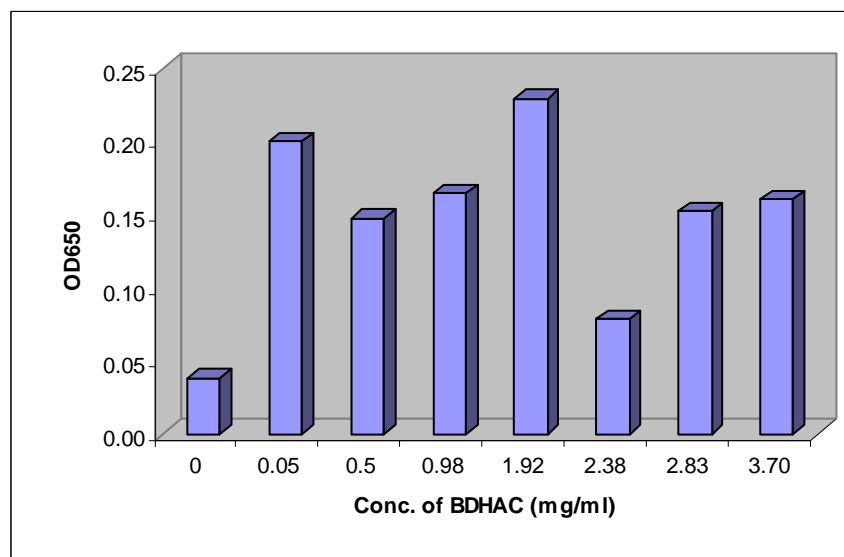


Figure 3.14 Optical Density as a Function of BDHAC Concentration for *Staphylococcus equorum* on Day 28 of Incubation.

Growth levels generally increased by day 28 of incubation at 0.05 and 1.92 mg/ml BDHAC compared to the control. Increased growth levels were also observed at 0.5, 0.98, 2.83 and 3.70 mg/ml BDHAC when compared to the control (Figure 3.14). The different concentrations of BDHAC in the growth medium supported the growth of this strain and the Quat may have been utilised by the strain as a source of carbon and energy for growth.

Sporosarcina sp.

Growth of the strain is shown on Figures 3.15 and 3.16. Steady growth levels were observed for BDHAC concentrations of 0.5 and 2.83mg/ml throughout the incubation period. At BDHAC concentration of 1.92 mg/ml, growth levels increased for the first 21 days of incubation and decreased by day 28. The sharp decrease in growth level may have been as a result of inadequate assimilation of organic substrate in the growth medium by the bacterium.

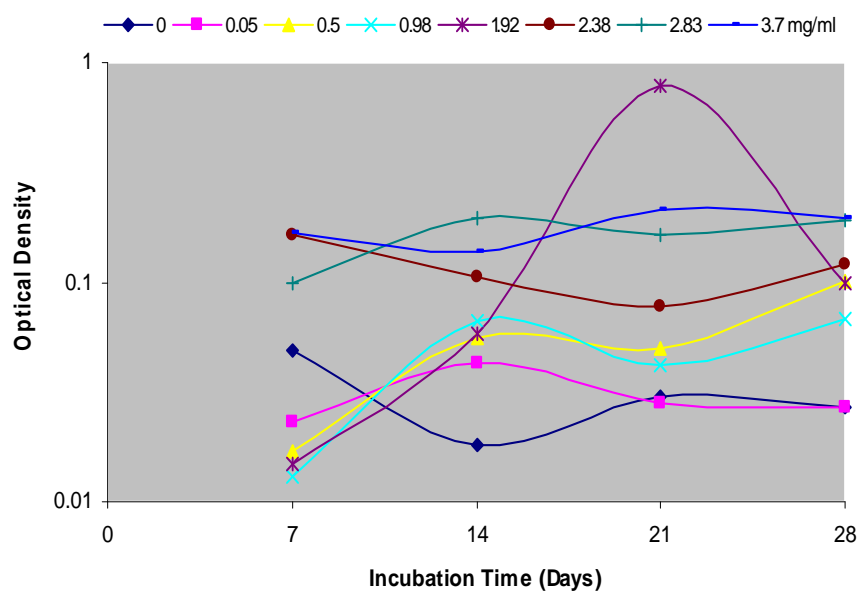


Figure 3.15 Growth of *Sporosarcina sp.* at Different BDHAC Concentrations.

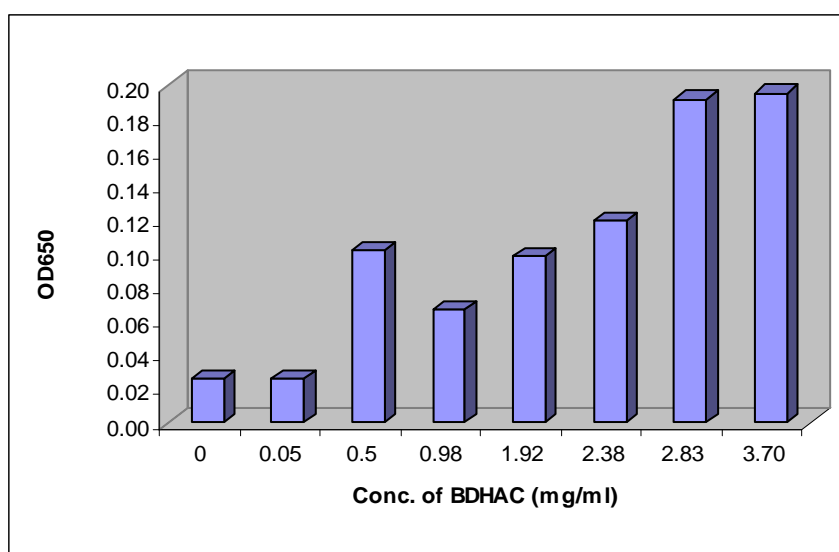


Figure 3.16 Optical Density as a Function of BDHAC Concentration for *Sporosarcina sp.* on Day 28 of Incubation.

Generally on day 28 of incubation, increase in growth levels were recorded for BDHAC concentrations 2.83 and 3.70 mg/ml when compared to the control. High growth levels were also observed for 0.5, 1.92 and 2.38 mg/ml of the Quat when compared to the control that lacked the test substance. It was interesting to observe that a high concentration of BDHAC such as 3.70 mg/ml did not inhibit the growth of the strain when BDHAC was present in the growth medium as a source of carbon and energy. Results obtained from the growth experiment revealed that the control that lacked BDHAC supported very limited growth of the strain and that the strain needed an organic substrate such as BDHAC for its growth.

3.4.2 Preparation of Calibration Curve

Results for the preparation of a calibration curve are summarised in Table 3.1. Absorbance values were recorded at a wavelength of 450nm and a calibration curve (Figure 3.17) was plotted relating absorbance to BDHAC concentration (mg/l). The curve showed linearity ($R^2 = 0.976$) signifying a good detection limit of the spectrophotometer. The equation of the

line generated from the calibration curve was used in calculating the analyte concentrations in the experimental samples.

Table 3.1 Concentration and Absorbance Values for the Plotting of a Calibration Curve

| Concentration of Std (mg/l) | Absorbance |
|-----------------------------|------------|
| 20 | 2.861 |
| 10 | 1.824 |
| 5 | 0.925 |
| 2 | 0.304 |

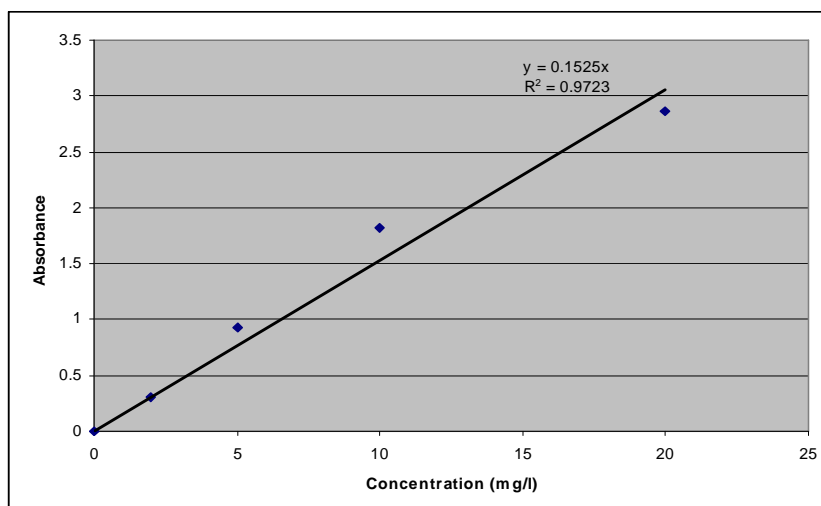
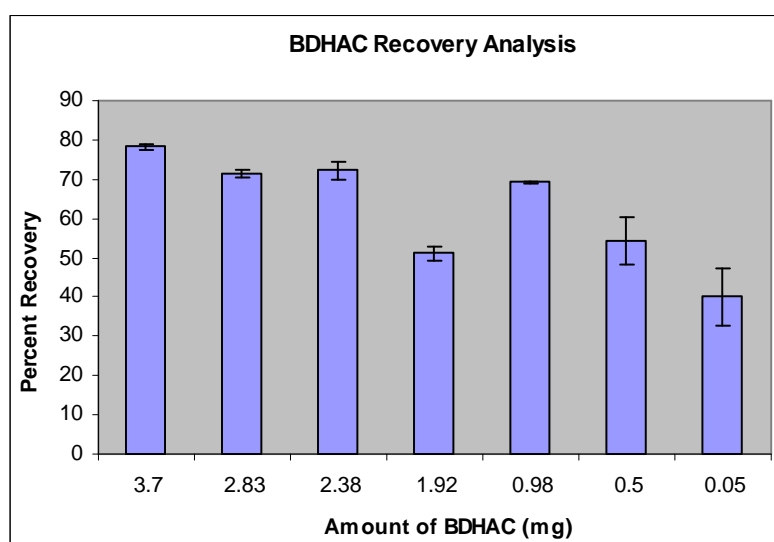


Figure 3.17 Calibration Curve for Dye Binding Assay

3.4.3 BDHAC Recovery Analysis

Quaternary ammonium compounds adhere to the surface of glassware during experiments (Swisher, 1970). Therefore to account for these losses due to adhesion to glassware, a recovery experiment was carried out to determine the actual amount of Quat removed due

to adsorption to glassware. The recovery experiment was carried out following the same procedure as the actual experiment but without incubation and was left for 24 hrs. The results obtained from the experiment were then used to correct calculated degradation values obtained from the actual experiment so as to ascertain whether the removal of BDHAC from the incubation mixture was due to biodegradation. The absorbance values obtained from the recovery experiment were related to the calibration curve (Figure 3.17) to obtain the amount of BDHAC in the sample. Sample percent recovery calculation is shown in Appendix A.2



Error margin = ± 1 Standard Deviation S.D (n = 2 extractions)

Figure 3.18 Percent Recovery from Flasks Containing Different Concentrations of BDHAC

Results for QAC recovery analysis are presented in Table 3.2 and illustrated in Figure 3.18. The analysis was carried out in duplicate and was reproducible. High mean percent recoveries were recorded for BDHAC concentrations of 3.7, 2.83, 2.38 and 0.98 mg/ml while low recovery was observed at BDHAC concentrations of 0.05 mg/ml. It was surprising to recover more amount of BDHAC at 0.98 mg/ml than at 1.92 mg/ml. Generally, results obtained from the recovery analysis by CDDBA suggested that high

amounts of residual BDHAC can be recovered from solutions of high concentrations of the Quat.

3.4.4 Quantification of Residual BDHAC by Colorimetric Dye Binding Analysis (CDBA)

From the Dye Binding Assay, absorbance values were recorded for residual BDHAC present in cultures of the various bacterial isolates incubated with different concentrations of the Quat and these values were inserted in the equation obtained from the calibration curve (Figure 3.17). The apparent extent of degradation of the QAC was determined and corrected by the losses shown by the recovery experiment, to estimate the quantity of BDHAC which had been degraded as a result of bacterial growth. Sample calculations showing amount of residual BDHAC, and degradation values are shown in Appendix C.

Analysis of BDHAC Degradation by the Bacterial Isolates: Analysis of BDHAC degradation by the different strains of bacteria is presented here.

Micrococcus luteus E

Table 3.2 BDHAC Amounts Added, Concentration and Residual Amounts after Biodegradation in Samples with *Micrococcus luteus* E from Day 28 of Incubation.

| BDHAC Amount (mg) | BDHAC Conc. (mg/ml) | Res. BDHAC Amount (mg) | % Degradation |
|----------------------|---------------------|---------------------------|---------------|
| 0.000 | 0.000 | - | - |
| 0.340 | 0.050 | 0.331 | 25.59 ±6.78 |
| 3.420 | 0.500 | 0.562 | 88.03 ±0.74 |
| 6.863 | 0.980 | 0.791 | 91.61 ±0.28 |
| 13.846 | 1.920 | 1.563 | 91.69 ±0.30 |
| 17.381 | 2.380 | 2.810 | 88.23 ±0.23 |
| 20.943 | 2.830 | 5.127 | 82.17 ±0.08 |
| 28.147 | 3.700 | 6.535 | 83.08 ±0.04 |

Table 3.2 show results from CDBA analysis of BDHAC on day 28 of incubation in the presence of *Micrococcus luteus* E. High percent degradation values (91.61 and 91.69) %

BIODEGRADATION OF BDHAC BY EIGHT STRAINS

were observed at concentrations of 0.98 and 1.92 mg/ml respectively. The lowest percent degradation was recorded for 0.05 mg/ml BDHAC.

A comparison of growth results to that of degradation for this strain, suggested that the presence of high amount of the Quat in the growth medium supported the growth of the strain as growth was shown to increase at high BDHAC concentrations. It was also deduced from the degradation results that the strain utilised BDHAC as a source of carbon and energy for growth.

Bacillus niabensis

Table 3.3 BDHAC Amounts Added, Concentration and Residual Amounts after Biodegradation in Samples with *Bacillus niabensis* from Day 28 of Incubation.

| BDHAC Amount (mg) | BDHAC Conc. (mg/ml) | Res. BDHAC Amount (mg) | % Degradation |
|----------------------|---------------------|---------------------------|---------------|
| 0.000 | 0.000 | - | - |
| 0.340 | 0.050 | 0.320 | 13.61±6.80 |
| 3.420 | 0.500 | 0.409 | 91.28 ±0.38 |
| 6.863 | 0.980 | 0.758 | 91.95 ±0.11 |
| 13.846 | 1.920 | 1.192 | 93.73 ±0.38 |
| 17.381 | 2.380 | 2.954 | 87.62 ±0.04 |
| 20.943 | 2.830 | 6.240 | 78.29 ±0.20 |
| 28.147 | 3.700 | 10.959 | 71.64 ±0.09 |

Results for the degradation of BDHAC by *Bacillus niabensis* on day 28 of incubation is shown in Table 3.3. The highest percent degradation value 93.73 % was observed at 1.92 mg/ml BDHAC. High percent degradation values (91.28, 91.95) % were also observed at 0.5 and 0.98 mg/ml BDHAC while the least percent degradation value 13.61% was seen at 0.05 mg/ml BDHAC.

A comparison of growth results to that of biodegradation for this strain revealed that high concentrations of BDHAC supported growth and an increased growth level was needed for rapid degradation of the Quat. Results obtained from the analysis of BDHAC in the presence of *Bacillus niabensis* on day 28 of incubation generally showed that high

BIODEGRADATION OF BDHAC BY EIGHT STRAINS

BDHAC concentrations of up to 3.70 mg/ml supported the growth of the strain when the Quat was present as a source of carbon and energy. The bacterium utilised BDHAC as an organic substrate for growth.

Bacillus subtilis

Table 3.4 BDHAC Amounts Added, Concentration and Residual Amounts after Biodegradation in Samples with *Bacillus subtilis* from Day 28 of Incubation.

| BDHAC Amount (mg) | BDHAC Conc. (mg/ml) | Res. BDHAC Amount (mg) | % Degradation |
|-------------------|---------------------|------------------------|------------------|
| 0.000 | 0.000 | - | - |
| 0.340 | 0.050 | 0.320 | 17.20 \pm 8.50 |
| 3.420 | 0.500 | 0.475 | 89.88 \pm 0.13 |
| 6.863 | 0.980 | 2.001 | 78.76 \pm 0.48 |
| 13.846 | 1.920 | 3.963 | 79.15 \pm 1.80 |
| 17.381 | 2.380 | 3.616 | 84.84 \pm 0.04 |
| 20.943 | 2.830 | 6.100 | 78.77 \pm 0.08 |
| 28.147 | 3.700 | 8.843 | 77.11 \pm 0.23 |

Table 3.4 show degradation results obtained on day 28 of incubation for *Bacillus subtilis*. High percent degradation values were observed at 0.5 and 2.38 mg/ml. The least percent degradation (17.20%) was at observed at 0.05 mg/ml BDHAC. Extents of BDHAC biodegradation were low at low concentrations of the QAC. Results from growth and degradation experiments at BDHAC concentrations of 2.38, 2.83 and 3.7 mg/ml, showed that increase in growth of *Bacillus subtilis* resulted in a corresponding increase in the extent of degradation. All the Quat concentrations tested, supported growth of the strain and the high extent of degradation observed also suggested that the bacteria had utilised the Quat as a source of carbon and energy.

Rhodospirillaceae

Table 3.5 BDHAC Amounts Added, Concentration, and Residual Amounts after Biodegradation in Samples with *Rhodospirillaceae* from Day 28 of Incubation.

| BDHAC Amount (mg) | BDHAC Conc. (mg/ml) | Res. BDHAC Amount (mg) | % Degradation |
|----------------------|------------------------|---------------------------|------------------|
| 0.000 | 0.000 | - | - |
| 0.340 | 0.050 | 0.180 | 61.58 \pm 3.41 |
| 3.420 | 0.500 | 0.755 | 83.89 \pm 0.38 |
| 6.863 | 0.980 | 2.259 | 76.25 \pm 0.45 |
| 13.846 | 1.920 | 6.559 | 65.49 \pm 0.19 |
| 17.381 | 2.380 | 7.306 | 69.36 \pm 0.10 |
| 20.943 | 2.830 | 7.452 | 74.00 \pm 0.08 |
| 28.147 | 3.700 | 16.375 | 57.61 \pm 0.07 |

BDHAC degradation results for *Rhodospirillaceae* on day 28 of incubation are presented in Table 3.5. The highest and the lowest percent degradation values (83.89 and 57.61) % were observed at 0.5 and 3.7 mg/ml BDHAC respectively. High percent degradation was also observed at 0.98 and 2.38 mg/ml of the Quat. Comparing growth of the strain to BDHAC degradation revealed that there was increase in growth at high concentrations of BDHAC but the increase did not result in high BDHAC degradation as was observed for *Bacillus subtilis* (Table 3.4). Generally, all the concentrations of BDHAC tested supported the growth of this strain of bacteria. The high extents of degradation observed showed that the strain was able to utilise up to 3.7 mg/ml BDHAC when it was present as a carbon and energy source in the growth medium.

Thalassospira sp.

Table 3.6 BDHAC Amounts Added, Concentration, Absorbance and Residual Amounts after Biodegradation in Samples with *Thalassospira sp.* from Day 28 of Incubation.

| BDHAC Amount (mg) | BDHAC Conc. (mg/ml) | Res. BDHAC Amount (mg) | % Degradation |
|----------------------|------------------------|---------------------------|------------------|
| 0.000 | 0.000 | - | - |
| 0.340 | 0.050 | 0.326 | 18.39 \pm 3.41 |
| 3.420 | 0.500 | 1.298 | 71.64 \pm 0.25 |
| 6.863 | 0.980 | 1.175 | 87.53 \pm 0.15 |
| 13.846 | 1.920 | 2.521 | 84.84 \pm 0.27 |
| 17.381 | 2.380 | 2.580 | 89.05 \pm 0.18 |
| 20.943 | 2.830 | 4.915 | 82.90 \pm 0.09 |
| 28.147 | 3.700 | 16.154 | 50.48 \pm 11.6 |

Results for the degradation of BDHAC at different concentrations by *Thalassospira sp.* is shown in Table 3.6. The extents of BDHAC biodegradation (50.48, 82.90 and 89.05 %) observed at 3.7, 2.83 and 2.38 mg/ml were high while low degradation values were observed at 0.05 and 0.5 mg/ml. For this strain, the highest level of degradation was not recorded for the highest BDHAC concentration which was a general trend for most of the strains.

A comparison of growth results for this strain to that from degradation, suggested that a low level of growth resulted in a high extent of degradation at 2.83 mg/ml BDHAC. This observation was different from those obtained for other strains at that concentration. All the BDHAC concentrations tested generally supported the growth of this strain and high levels of degradation at high concentrations showed that the strain was capable of utilizing BDHAC as a source of carbon and energy in the production of new cells.

Micrococcus luteus C

Table 3.7 BDHAC Amounts Added, Concentration, Absorbance and Residual Amounts after Biodegradation in Samples with *Micrococcus luteus* C from Day 28 of Incubation.

| BDHAC Amount (mg) | BDHAC Conc. (mg/ml) | Res. BDHAC Amount (mg) | % Degradation |
|----------------------|------------------------|---------------------------|------------------|
| 0.000 | 0.000 | - | - |
| 0.340 | 0.050 | 0.151 | 67.61 \pm 11.9 |
| 3.420 | 0.500 | 0.356 | 92.43 \pm 0.74 |
| 6.863 | 0.980 | 0.761 | 91.93 \pm 0.05 |
| 13.846 | 1.920 | 1.507 | 92.19 \pm 0.31 |
| 17.381 | 2.380 | 2.253 | 90.56 \pm 0.15 |
| 20.943 | 2.830 | 5.323 | 81.67 \pm 0.06 |
| 28.147 | 3.700 | 12.507 | 67.62 \pm 0.07 |

Results for the degradation of different concentrations of BDHAC by *Micrococcus luteus* C are shown in Table 3.7. The highest and lowest percent degradation were observed at 0.98 and 1.92 mg/ml BDHAC respectively. Results obtained revealed that the extent of BDHAC degradation decreased with a decrease in concentration of BDHAC in the growth medium. Comparing growth of the strain to BDHAC degradation suggested that the high extents of degradation observed was not as a result of increase in growth except at 2.38, 1.92 and 0.98 mg/ml BDHAC were growth resulted in degradation. The results generally showed that all the concentrations of BDHAC tested did not inhibit the growth of the strain during the 28 days of incubation and that the strain was able to utilise the Quat for the production of new cells.

Staphylococcus equorum Q

Table 3.8 BDHAC Amounts Added, Concentration, Absorbance and Residual Amounts after Biodegradation in Samples with *Staphylococcus equorum* from Day 28 of Incubation.

| BDHAC Amount (mg) | BDHAC Conc. (mg/ml) | Res. BDHAC Amount (mg) | % Degradation |
|-------------------|---------------------|------------------------|---------------|
| 0.000 | 0.000 | - | - |
| 0.340 | 0.050 | 0.133 | 64.01± 3.39 |
| 3.420 | 0.500 | 0.991 | 78.86± 2.50 |
| 6.863 | 0.980 | 1.237 | 86.87± 0.40 |
| 13.846 | 1.920 | 2.738 | 85.60± 0.16 |
| 17.381 | 2.380 | 2.542 | 89.34± 0.10 |
| 20.943 | 2.830 | 6.535 | 77.26± 0.05 |
| 28.147 | 3.700 | 7.482 | 80.63± 0.14 |

Results for the degradation of BDHAC by *Staphylococcus equorum* on day 28 of incubation are shown in Table 3.8. High percent degradation was observed at 0.98, 1.92 and 2.38 mg/ml of the Quat. It was surprising to observe that 2.38 mg/ml BDHAC was degraded more than 2.83 mg/ml. Other strains exhibited high extents of degradation at high concentrations. Also the highest extent of BDHAC degradation was not recorded at 1.92 mg/ml that supported the most growth. The highest extent of degradation was rather observed at a concentration with minimal growth of the strain. Quat degradation was shown to decrease with a decrease in concentration of BDHAC except at 2.38 mg/ml.

Results for growth and degradation for this strain showed that all the concentrations of BDHAC tested did not inhibit the growth of this strain and that it was able to utilise the Quat as an organic substrate to derive energy for growth during 28 days of incubation.

Sporosarcina sp. H

Table 3.9 BDHAC Amounts Added, Concentration, Absorbance and Residual Amounts after Biodegradation in Samples with *Sporosarcina sp.* from Day 28 of Incubation.

| BDHAC Amount (mg) | BDHAC Conc. (mg/ml) | Res. BDHAC Amount (mg) | % Degradation |
|-------------------|---------------------|------------------------|---------------|
| 0.000 | 0.000 | - | - |
| 0.340 | 0.050 | 0.331 | 18.40 ±6.80 |
| 3.420 | 0.500 | 0.351 | 92.87 ±0.12 |
| 6.863 | 0.980 | 0.522 | 94.47 ±0.14 |
| 13.846 | 1.920 | 7.454 | 60.76 ±0.36 |
| 17.381 | 2.380 | 6.459 | 72.93 ±0.05 |
| 20.943 | 2.830 | 15.113 | 47.70 ±0.20 |
| 28.147 | 3.700 | 16.206 | 58.04 ±0.59 |

Table 3.9 illustrates the results obtained from BDHAC degradation experiment on day 28 in the presence of *Sporosarcina sp.* High percent degradation was observed at 0.5 and 0.98 mg/ml BDHAC respectively. Also, low percent degradation was observed at 0.05 and 2.83 mg/ml.

The extents of BDHAC degradation was shown to decrease with decrease in concentration of the Quat in the growth medium except at 2.83 mg/ml where the extent of degradation was close to those recorded for 0.98 and 1.92 mg/ml. A Comparison of growth results to extents of BDHAC biodegradation, suggested that high growth levels led to high extent of degradation at 2.38, 2.83 and 3.70 mg/ml BDHAC. The overall growth and degradation results showed that 3.70 mg/ml of the Quat was not toxic to the strain as it was able to survive in a growth medium containing the Quat.

3.5 Discussion

Results for growth and degradation of BDHAC by 8 strains of bacteria during 28 days of incubation revealed that up to 3.70 mg/ml of the Quat i.e the maximum concentration of BDHAC used, supported the growth of the strains. A general trend observed for most of the strains was that the highest and lowest extents of BDHAC degradation were at concentrations of 3.70 and 0.05 mg/ml. Also there was an increase in growth levels for all the strains at 1.92, 2.38, 2.83 and 3.70 mg/ml on the 28th day of incubation suggesting that these concentrations of BDHAC did not inhibit the growth of the strains.

Micrococcus luteus E and C, *Bacillus niabensis* G, and *Staphylococcus equorum* Q, showed high growth levels at 1.92 mg/ml BDHAC. Also at 2.38, 2.83 and 3.70 mg/ml of the Quat, *Micrococcus luteus* E, *Bacillus subtilis* F, *Rhodospirillaceae* K and *Sporosarcina* sp. H showed increased growth levels. Generally growth of the strains was shown to increase by day 28 of incubation when compared to the first 21 days. BDHAC analysis by CDBA showed that high amounts of the Quat can be recovered from its solution at high concentrations. The recovery experiment showed that BDHAC recovery was proportional to the starting concentration. *Micrococcus luteus* E showed the greatest ability to degrade 3.7 mg/ml BDHAC when compared to other strains. This was closely followed by *Staphylococcus equorum* Q, *Bacillus subtilis* F, *Bacillus niabensis* G and *Micrococcus luteus* C. It was interesting to observe that *Thalassospira* sp. J, *Sporosarcina* sp. H and *Rhodospirillaceae* K degraded 3.7 mg/ml by almost the same level. The extents of Quat degradation observed for all the strains supports the observations that regular exposure to QACs of Gram-positive and Gram-negative bacteria bring about their adaptation and resistance to these chemicals (Russell et al. 1986 and Sundheim, et al., 1992). Comparing growth results to that of degradation for the different strains suggested that the extent of BDHAC degradation for some of the strains was not proportional to growth levels observed. Slow growth of bacteria is as a result of a long lag phase or extended period of acclimation before assimilation of substrate present in the growth medium as a source of carbon and energy.

For *Micrococcus luteus* C and E, *Bacillus subtilis* F, and *Bacillus niabensis* G, BDHAC degradation was observed to decrease with a decrease in the concentration of Quat

in the growth medium. The strains were able to utilise the substrate however, but growth and degradation were not the same for all the bacteria. A bacterium grows by assimilating nutrients from substrates present in the growth medium and producing new cells by cell division. The decrease in the concentration of BDHAC originally present in the growth medium was as a result of possible degradation by the different strains of bacteria. Alexander, (1999) reported that during a typical growth-linked mineralisation by bacteria, the cells use some of the energy and carbon of their organic substrate to make new cells and this increasingly large population causes rapid mineralisation. Also, findings from this research corroborate those of Ventullo and Larson (1986) and Vesteege and Shorter (1992) on the adaptation to QACs by aquatic organisms through their repeated exposure to these compounds and the biodegradation of QACs by pure cultures of bacteria.

3.6 Conclusion

This study showed that BDHAC at concentrations of 0.05, 0.5, 0.98, 1.92, 2.38, 2.83 and 3.70 supported the growth of 8 strains of bacteria. High extents of degradation observed on day 28 of incubation also showed that the Quat was not toxic to these strains of bacteria. Although growth levels were minimal, all the results supported the observation that the bacteria were capable of utilising BDHAC as an organic growth substrate in the growth medium. Four of the strains were investigated further in the next section to ascertain the extent at which the Quat can be degraded and also the availability of possible degradation products.

SECTION B

BIODEGRADATION OF BDHAC BY FOUR STRAINS OF BACTERIA

This section investigates four strains of bacteria selected from those previously considered in section A. The selection was based on the observation in section 3.4.1, four strains of bacteria identified as *Bacillus niabensis*, *Sporosarcina sp.*, *Bacillus subtilis*, and *Thalassospira sp.* were shown to grow to some extent at BDHAC concentrations of 1.92 and 3.70mg/ml. It was important to perform further, more detailed experiments so as to determine the extent to which these strains can grow in the media containing BDHAC and utilise it when it was present at different concentrations.

3.7 Introduction

From the results presented in Section A, four strains of bacteria were selected for a more detailed investigation. The study was carried out on a larger scale than that reported in Section A i.e. ten times the culture volumes and slightly increased concentrations. The increase in volume was to ensure that enough samples and materials were available for identification of possible metabolites of QAC degradation. Analysis of residual BDHAC was performed using CDBA and the presence of metabolic products was detected by ESI-MS in the positive mode (results presented in Chapter 4).

3.8 Aim of the Study

This study, utilising Benzyldimethyl Hexadecylammonium Chloride [BDHAC] investigated the utilisation of the surfactant by four selected strains of heterotrophic bacteria at slightly increased concentrations and checked for possible degradation products formed in degradation experiments by the procedures of Patrauchan and Oriel (2003).

3.9 Materials and Methods

Reagents used were analytical grade and were as described in section 3.3

Preparation of Growth Medium

Preparation of media was performed following the procedures described in section 3.3.1 but the volume was increased ten times in order to have enough sample volume for the investigation of possible products of metabolism of the QAC. A marine broth incolum was used which exposed the strains of bacteria to 1 mg/ml BDHAC before a final inoculation in a batch culture was carried out. This was necessary to expose the strains to BDHAC so that growth and degradation will be enhanced in the batch culture during the 28 days incubation. Regular exposure to chemicals such as QACs of bacteria brings about their adaptation and resistance to these chemicals (Sundheim et al., 1992)

Preparation of Standards

5 g of BDHAC was dissolved in methanol in a 100ml volumetric flask (**Standard 1**). From the stock standard, concentrations of 20mg/20 ml, 200mg/100 ml and 400mg/100 ml were then prepared and used as standards for measuring BDHAC concentration.

BDHAC Degradation Experiment

Strains from the frozen stock were streaked out on marine agar plates and allowed to grow to pure, single colonies. Marine broth solution was prepared in four 20 ml universal bottles and autoclaved to sterilise the medium. The four bottles were later divided into two groups, each group having two bottles containing marine broth media. To these groups, 20 ml solution containing 1mg/ml BDHAC was added and they were inoculated with single colonies of *Bacillus niabensis*, *Sporosarcina* sp., *Bacillus subtilis*, or *Thalassospira* sp. and incubated for 5 days at 28°C. The cultures were then centrifuged and cells were resuspended in growth medium. The cells were re-centrifuged and resuspended in a small

volume (5ml) of medium (inoculum) and optical density was determined at 650nm. The re-centrifuging and re-suspension steps above were carried out to remove all the traces of marine broth.

For optical density measurement, 0.2ml of the inoculum was diluted with 1.8ml of medium (x 10 dilutions) and the OD of the resulting suspension was recorded. Given the value of the OD as X, the volume of the inoculum added to 100ml of medium in a 250 ml flask was calculated by taking $V = 0.08/X \times 100\text{ml}$ where V= volume of the inoculum, 0.08 is the required starting OD value of the 100 ml culture, X is the OD value of the inoculum, and 100 ml is the volume of growth medium.

Four 250ml flasks containing 100 ml of growth medium with different BDHAC concentrations i.e. 2 mg/ml and 4 mg/ml were inoculated with the required volumes of inocula in the range of 1.95-3.05 ml, calculated to give a starting OD value of around 0.08. *Bacillus niabensis* and *Sporosarcina sp.* were contained in flasks with 2 mg/ml of BDHAC while *Bacillus subtilis* and *Thalassospira sp.* were in flasks with 4 mg/ml BDHAC. This was based on growth results obtained in section 3.4.2 which suggested optimum growth of the strains at these concentrations. The experiment was carried out in duplicate.

The flasks were incubated on a shaker at 120 rev/min, at 25°C, for 28days. On days 0, 7, 14, 21 and 28, 1ml samples were taken out for optical density determination and electrospray ionisation mass spectrometric analysis while 5ml was taken out for the colorimetric dye binding assay. The total volume taken out from the flasks at each time was 7ml, bringing the total volume removed from each flask to 35ml after 28days of incubation.

Analytical Methods

ESI-MS/MS and CDBA were used for quantification of residual BDHAC while SPSS version 16.0 was used for statistical analysis.

Quantification of Residual BDHAC by Colorimetric Dye Binding Assay (CDBA)

Preparation of calibration curve: 50mg of BDHAC was dissolved in methanol in a 50ml volumetric flask resulting in 1mg/ml or 1000mg/l standard (**std.1**). 1.0, 0.5, 0.25 and 0.1 ml of **std.1** were added to 5 different 50ml volumetric flasks with growth medium and the resulting concentrations were 20, 10, 5 and 2 mg/l. A calibration curve was prepared following the method described in Chapter 2 (section 2.3.7)

5ml was taken from the culture and centrifuged to remove cells before extraction with 10 ml DCM in a separating funnel. 1ml from the 10 ml DCM extract was blown to dryness under a gentle stream of nitrogen and the residual Quat made up to the mark in seawater in a 50 ml volumetric flask. The contents of the flasks were analysed following the procedures described in Chapter 2 (section 2.3.7)

BDHAC Recovery Analysis

To account for losses of BDHAC due to adsorption to glassware, a recovery experiment was carried out. For the recovery experiment, two 250ml flasks with 100ml of growth medium were required. 4 ml of 50mg/ml Quat stock standard solution was added to one flask to give a concentration of 2mg/ml and 8ml of 50mg/ml Quat standard solution was added to the other to give a concentration of 4mg/ml. The flasks were left for 28 days and on the 28th day, 5.0 ml was removed from the flasks and used for the recovery experiment following the procedures described in Chapter 2 (section 2.3.7)

3.10 Results

Physiological Characterisation and Biochemical Tests on Bacterial Strains.

After the experiment had been set up to run for 28 days, some screening tests were carried out on the bacterial strains to ensure that there was no change in their characteristics while being stored at -75°C in the freezer. Table 3.10 summarises the results of the tests that were

carried out. Details of these tests are discussed in Chapter 2. As expected, there was no change in characteristics of the strains.

Table 3.10 Physiological Characteristics and Biochemical Tests on Bacterial Strains

| Strains | Catalase Test | Oxidase Test | Gram Stain | Motility Test | Shape |
|---------------------------|---------------|--------------|------------|---------------|-------|
| <i>Bacillus subtilis</i> | + | + | Gram +ve | + | Rods |
| <i>Bacillus niabensis</i> | ++ | + weak | Gram +ve | + | Rods |
| <i>Sporosarcina sp.</i> | ++ | + | Gram –ve | + | Rods |
| <i>Thalassospira sp.</i> | + | + | Gram –ve | + | Rods |

Optical Density Measurement for the Different Bacterial Strains

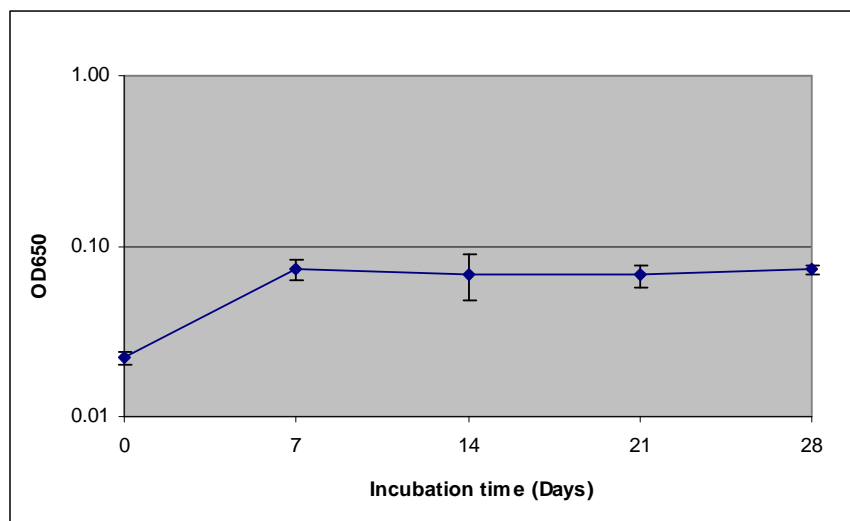


Figure 3.19 Growth of *Bacillus niabensis* in Medium Containing 2 mg/ml BDHAC

In a growth medium containing 2 mg/ml BDHAC with *Bacillus niabensis* results revealed that growth increased by Day 7 with an OD value slightly above 0.07 (Figure 3.19). Growth level remained constant between Days 7 and 28. The steady level of growth observed for this strain indicated that it survived in 2 mg/ml BDHAC. Growth of the strain in the previous experiment (section A) was in agreement with this result in that at the same concentration, there was increase in growth from the first seven days until the last day of incubation (Figure 3.3). Generally, growth result for this strain showed that BDHAC supported its growth when it was present at a concentration of 2 mg/ml.

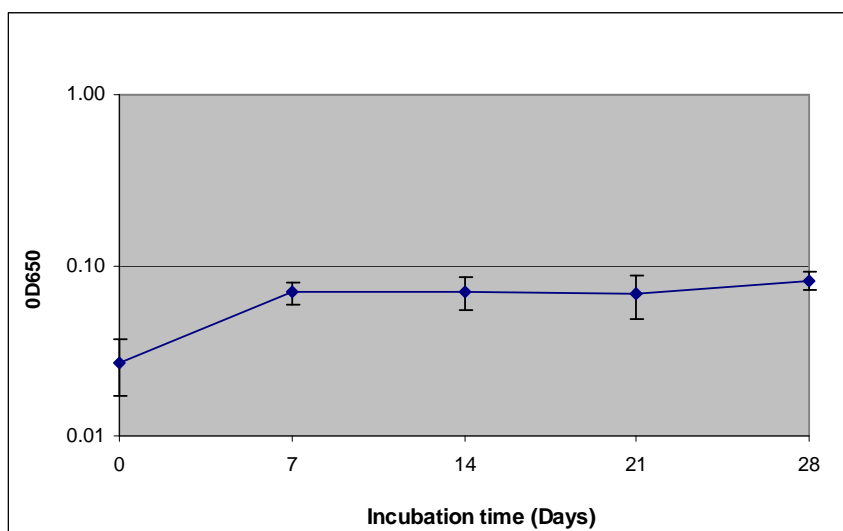


Figure 3.20 Growth of *Bacillus subtilis* in Medium Containing 4 mg/ml BDHAC

Growth of *Bacillus subtilis* reached an OD value of 0.07 by Day 7 of incubation. Growth stayed relatively constant after the first 7 Days of incubation (Figure 3.20). The concentration of BDHAC in the growth medium was 4 mg/ml and this concentration did not inhibit the growth of this strain. When growth results were compared to that obtained from previous experiments involving this bacterium (Section A, Figure 3.5), the difference was that growth decreased by Day 21. Steady growth observed here may be attributed to

the adaptation of the strain to BDHAC from an initial exposure of the strain to the QAC prior to the biodegradation experiment.

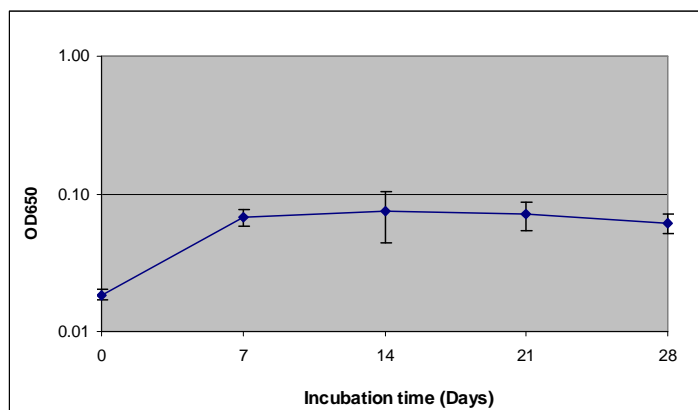


Figure 3.21 Growth of *Sporosarcina sp.* in Medium Containing 2 mg/ml BDHAC

Figure 3.21 illustrates the growth of *Sporosarcina sp.* during 28 days of incubation in culture with 2 mg/ml BDHAC. There was an increase in growth of the strain as the experiment progressed. Growth increased in the first 14 days of incubation and remained steady until Day 28. This result was not consistent with that obtained in the previous section (Figure 3.15) because growth was shown to increase after the first 21 days. However, growth results for this strain generally suggested that the strain survived and grew in the presence of 2 mg/ml BDHAC when it was present as a source of carbon.

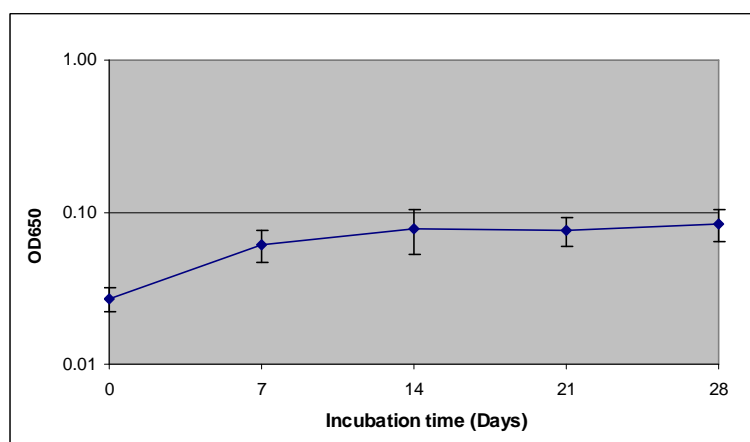


Figure 3.22 Growth of *Thalassospira sp.* in Medium Containing 4 mg/ml BDHAC

Figure 3.22 illustrates the growth of *Thalassospira sp.* cultivated in a growth medium containing 4 mg/ml BDHAC. There was gradual increase in growth by Day 7 where OD reached 0.06. Growth was seen to further increase after which it reached a kind of plateau by Day 21 and slightly increased again. This result was consistent with that observed for the bacterium in section A (Figure 3.9) in that increase in growth continued until the last day of incubation. Based on this result, it was deduced that 4 mg/ml of the QAC was not toxic to the bacteria.

Generally, as was observed in Section A for all the strains, BDHAC concentrations of 2 and 4 mg/ml were shown to support growth. At the start of the experiment, the strains were initially exposed to BDHAC prior to inoculation with the aim of obtaining a starting OD of 0.08, so as to ascertain that the cells were growing rapidly in the medium. However, it appeared the strains went into a short acclimation period and growth wasn't as fast as was expected. However, this finding supports the results obtained in Section A, which revealed that up to 3.7 mg/ml (approximately 4 mg/ml) BDHAC could support the growth of bacteria in 28 days of incubation.

BDHAC Recovery Analysis

QAC's strongly adsorb to glass surfaces of test containers, to natural solids such as clay, to bacterial cell walls, and to humic materials (Swisher 1970). Therefore, it is difficult to distinguish removal due to adsorption from that due to biodegradation, a situation that may account for the wide range of biodegradabilities reported (0-100%) for structurally similar QAC's (Swisher, 1970). For this reason, BDHAC recovery analysis was performed and the results are shown in Table 3.11

Table 3.11 Percent BDHAC Recovery Values from Experiment

| Concentration (mg/ml) | Absorbance | % Recovery |
|-----------------------|---------------|--------------|
| 4 | 0.652 ± 0.001 | 93.10 ± 0.02 |
| 2 | 0.369 ± 0.002 | 88.35 ± 0.01 |

Results from the recovery experiment showed 93.10% recovery of sample initially containing 4mg/ml BDHAC and 88.35% of sample with 2mg/ml of the test substance. Therefore, in this study, those values from the recovery experiment were used to correct the values obtained from the cultures examining degradation of BDHAC. Representative calculations for percent recovery and degradation values are shown in Appendix A.3.

Quantification of Residual BDHAC by Colorimetric Dye Binding Assay (CDBA)

Results for the quantification of residual BDHAC in growth medium by CDBA are presented here.

Preparation of Calibration Curve

Table 3.12 Concentration and Absorbance Values Used to Construct a Calibration Curve.

| Concentration of Std (mg/l) | Absorbance |
|-----------------------------|------------|
| 20 | 2.860 |
| 10 | 1.825 |
| 5 | 0.927 |
| 2 | 0.306 |

Table 3.12 shows the results of the calibration curve. Absorbance values for 2-20mg/l BDHAC were within the detection limit of the spectrophotometer (3.000) and were plotted against their corresponding concentrations to obtain a calibration curve (Figure 3.23).

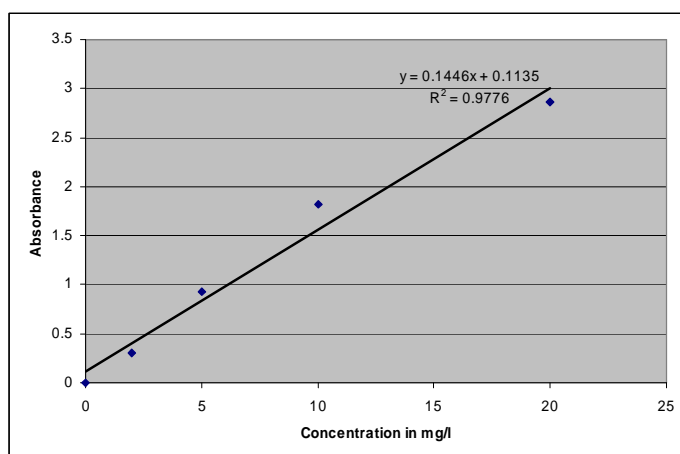
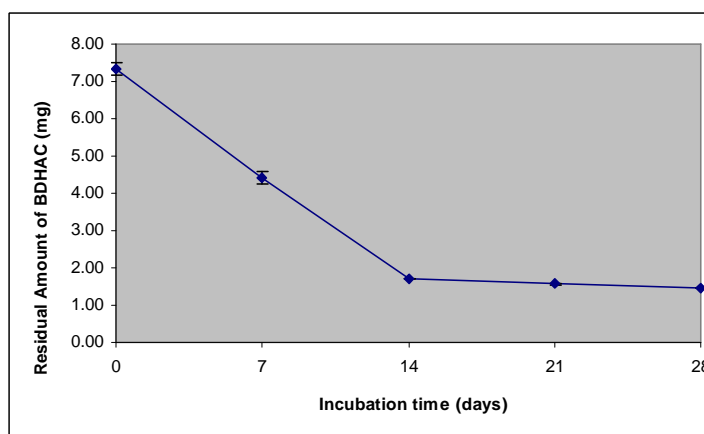


Figure 3.23 Calibration Curve for Calorimetric Dye Binding Assay

($Y = 0.1446x + 0.1135$ $Y = \text{absorbance}$; $X = \text{concentration of BDHAC in mg/l}$)

The curve showed a low detection limit for the instrument based on its linearity ($R^2 = 0.9776$). A straight line equation of the line was generated and was used in the calculation of the concentration of residual BDHAC after biodegradation on days 0, 7, 14, 21 and 28 of incubation. Results for the quantification of residual BDHAC by CDBA are discussed accordingly. Sample degradation calculations are shown in Appendix D.1

Bacillus niabensis.

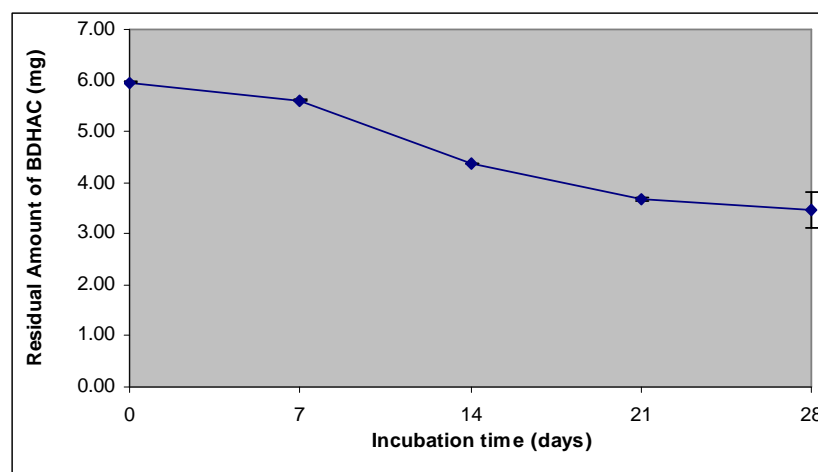


Error bars represents ± 1 (SD) and $n = 2$ extractions

Figure 3.24 Degradation of BDHAC by *Bacillus niabensis*. as Determined by CDBA

Figure 3.24 show the degradation of 2 mg/ml BDHAC by *Bacillus niabensis*. The amount of residual BDHAC decreased with incubation time for the first 14 Days of incubation. Between Day 14 and 28, the extent of BDHAC degradation was steady. Growth results (Figure 3.19) for the strain was in agreement with the extent of BDHAC degradation recorded. Generally, growth was seen to increase as the amount of BDHAC decreased. This observation suggests that 2 mg/ml of BDHAC in the growth medium did not inhibit the growth of *Bacillus niabensis* and the strain must have utilised the BDHAC as a source of carbon and energy.

Bacillus subtilis.

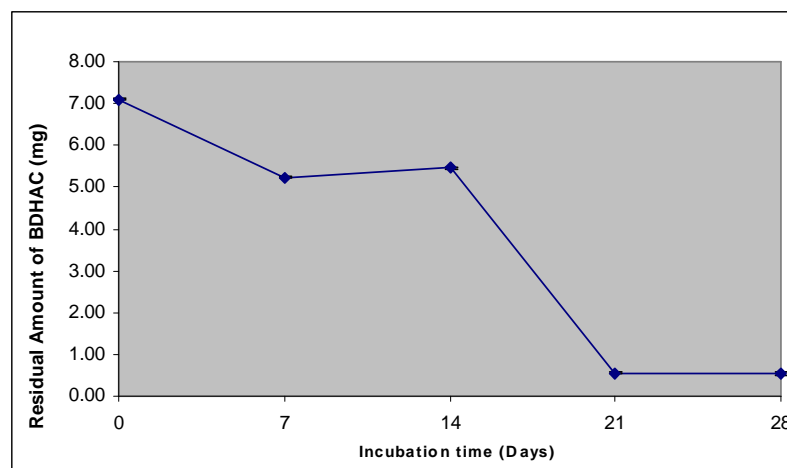


Error bars represents ± 1 (SD) and n= 2 extractions

Figure 3.25 Degradation of BDHAC by *Bacillus subtilis*. as Determined by CDBA

Results for the degradation of 4 mg/ml BDHAC by *Bacillus subtilis* are shown in Figure 3.25. The amount of residual BDHAC decreased with incubation time. The decrease was gradual over the 28 days of incubation. Generally, the results obtained were in agreement with growth results obtained for this strain (Figure 3.20) and suggest that *Bacillus subtilis* present in the growth medium could utilise 4mg/ml BDHAC.

Sporosarcina sp.

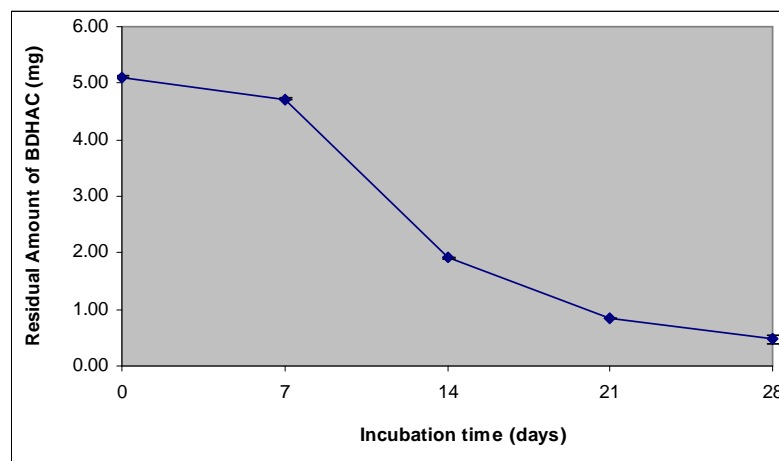


Error bars represents ± 1 (SD) and n= 2 extractions

Figure 3.26 Degradation of BDHAC by *Sporosarcina sp.* as Determined by CDBA

Figure 3.26 show the amount of residual BDHAC from a growth medium with *Sporosarcina sp.* The amount of residual BDHAC decreased with incubation time. A large decrease in the amount of BDHAC was observed between Days 14 and 21. Generally, it was deduced that BDHAC at a concentration of 2 mg/ml did not inhibit the growth of *Sporosarcina sp.* and that a large amount of BDHAC was lost between Days 14 and 21 of incubation suggesting that growth was accompanied by biodegradation.

Thalassospira sp.



Error bars represents ± 1 (SD) and n= 2 extractions

Figure 3.27 Degradation of BDHAC by *Thalassospira sp.* as Determined by CDBA

Figure 3.27 illustrate the results obtained from the degradation experiment with 4 mg/ml BDHAC in the presence of *Thalassospira sp.* In this case, there was a large decrease in the amount of BDHAC in the growth medium by Day 14. The decrease continued until Day 21 and the concentration of BDHAC remained stable until day 28. The results obtained revealed that 4 mg/ml BDHAC was not toxic to *Thalassospira sp.*, but that the strain showed some level of growth in the medium at that concentration of BDHAC (Figure 3.22). This observation suggests the utilisation of BDHAC by *Thalassospira sp.* as a carbon and energy source.

Generally, the results obtained from the analyses of residual BDHAC after degradation by *Thalassospira sp.*, *Bacillus subtilis*, *Sporosarcina sp.* and *Bacillus niabensis* by CDBA indicated that the bacteria were capable of degrading BDHAC when present at concentrations of 2 or 4mg/ml. Although losses in the amount of BDHAC were observed for Day 0, it may have been as a result of centrifugation that was carried out prior to extraction with DCM. In the process, BDHAC bound to cell materials may have been

removed thereby reducing the amount of the QAC. The surface charge of the strains was not measured. Based on the findings from this Chapter, it would be important to investigate further the extent of utilisation of BDHAC by these strains of bacteria at much lower concentrations and incubation time (hours) to check if higher extents of growth and degradation would be evident.

3.11 Discussion

This research reports the utilisation of BDHAC by four bacterial strains isolated from marine sediments. The concentrations of BDHAC utilised by these strains of bacteria were slightly higher than those earlier reported in section A. Continuous exposure of the strains to BDHAC in marine broth inoculum and the biodegradation experiment resulted in uniform growth of the strains in the growth medium. Many researchers have reported adaptation to QACs by aquatic organisms via their continuous exposure to these compounds (Versteeg and Shorter, 1992; Takenaka, 2007) and the biodegradation of QACs by pure cultures of bacteria (Ventullo and Larson, 1986). McBain et al. (2004) also showed that repeated exposure of pure cultures of bacteria to QAC altered their susceptibility to these compounds. Also higher recoveries of BDHAC were recorded for this section compared to previous experiments, suggesting that losses observed were due to biodegradation and not adsorption of the surfactant to test flasks.

Generally, there was increase in growth as the experiment progressed. Although there was difference in the extent of BDHAC degradation observed for all the strains, the important observation was that biodegradation accompanied growth. Results obtained have suggested that consortia of micro-organisms may not be required for the degradation of quaternary ammonium salts at least at primary level, but rather that pure cultures of bacteria were capable of degrading BDHAC. The degradation of BDHAC by pure cultures of *Thalassospira sp.*, *Bacillus subtilis*, *Sporosarcina sp.* and *Bacillus niabensis*, have implications both for natural degradation of BDHAC in marine sediment and bioremediation of contaminated sediment.

Bioremediation is the process that uses microorganisms, or their enzymes to return the natural environment altered by contaminants to its original condition. The technique

may be employed to attack specific contaminants, such as degradation of QACs by bacteria (Meagher, 2000). Generally, bioremediation technologies can be generally classified as *in situ* or *ex situ*. *In situ* bioremediation involves treating the contaminated material such as sediment at the site while *ex situ* involves the removal of the contaminated material to be treated elsewhere. Examples of bioremediation technologies are biostimulation, bioaugmentation, bioventing, landfarming, bioreactor, composting, and rhizofiltration, (Meagher, 2000).

Bioremediation can occur by natural attenuation or can be spurred on via the addition of nutrients to increase bioavailability within a medium (biostimulation). Also, addition of matched strains of microbes to a medium to enhance the resident microbe population's ability to break down contaminants (bioaugmentation) is another form of bioremediation (Diaz, 2008).

From the findings of this research, it would be worth investigating if these strains of bacteria can quickly utilise smaller concentrations of BDHAC to support growth judging from the slow growth that was observed in this section. Based on this, further investigation that will be carried out in the next section will focus on the addition of smaller concentrations of BDHAC to the growth medium and a further reduction in the incubation time for the experiment. Also efforts would be made at identifying other possible products of BDHAC metabolism.

3.12 Conclusion

In summary, this study showed that four strains of bacteria were capable of degrading the corrosion inhibitor active BDHAC to a large extent when it was the only carbon source present in the growth medium. The study showed that different types of bacteria have the ability to degrade BDHAC.

SECTION C

BIODEGRADATION OF BDHAC BY THE FOUR STRAINS OF BACTERIA INVESTIGATED IN SECTION B AND INCUBATED IN MARINE BROTH MEDIA.

3.13 Introduction

This study was a follow up of the investigation carried out in Section B but with the following modifications: a) a change in the growth medium used for the cultivation of the strains of bacteria to investigate whether this would result in greater growth of the bacteria in the presence of BDHAC, and better degradation and generation of breakdown products for analysis;

b) Exposure of the bacteria to lower concentrations of BDHAC (0.1 mg/ml, 0.25 mg/ml, 0.5 mg/ ml and 1mg/1ml) of BDHAC.

c) The incubation time for the degradation studies was kept at 120 hours.

3.14 Aim of the Study

This study investigated whether bacteria could grow better when cultivated in marine broth compared to minimal salt media, and with lower concentrations (0.1, 0.25, 0.5, and 1.0mg/ml) of BDHAC. Results from section B showed minimal growth of the QAC in the presence of 2 and 4 mg/ml BDHAC. This study will check if the strains can grow better in a different growth medium and reduced BDHAC concentrations.

3.15 Materials and Methods

All the reagents used were analytical grade and were as described in Section A of this Chapter.

Preparation of Media

Marine Broth Medium was prepared by dissolving 37.4g of Marine Broth (Difco, Fisher Scientific Co. Ltd. UK.) in one litre of sea water.

Preparation of Standards

5g of BDHAC was dissolved in methanol in a 100ml volumetric flask. From stock standard, final concentrations prepared in growth media were 0.1 mg/ml, 0.25 mg/ml, 0.5 mg/ml and 1mg/ml of BDHAC.

Determination of the Effect of Methanol on the Growth of Bacterial Strains

The growth of the strains of bacteria in the previous chapter was slow and it was important to check for possible effect of methanol used as a solvent for the BDHAC on growth of the strains. The volume of methanol (2 ml) chosen for this study was that volume that would be contained in the medium containing the highest BDHAC concentration (1mg/ml). The methanol toxicity experiment was performed following steps 1 and 2 described below.

Step 1

From the frozen stock in the freezer, strains were streaked out on marine agar plates and were allowed to grow to single colonies. Four universal bottles containing 20ml of marine broth were autoclaved at 121°C before being inoculated by single colonies of bacteria from the agar plates. The samples were incubated at 25°C for 120 hrs before optical Density was measured. The final Optical Density value that was required for Step 2 of the experiment was 0.08 in 100ml of growth media, so a measured volume i.e. 0.25ml was taken out of the 20ml culture and was diluted four times with 10% formalin. Optical density of the sample was then measured at 650 nm to obtain an initial OD value X, therefore $OD \times 4 = X$. The volume added to the 100 ml solution in 250ml flasks was calculated using the relation $V = 0.08/X \times 100$ where X is the final OD value, and 0.08 the initial OD value in the next culture.

BIODEGRADATION OF BDHAC BY FOUR STRAINS INCUBATED IN MARINE BROTH

Step 2

16 flasks, each containing 100ml of marine broth, were autoclaved and subsequently divided into two main groups, namely: Group A, and B, each group having 8 flasks. Thereafter 2ml of methanol was added to 100ml of the marine broth solution in Group B followed by the inoculation of flasks in both Groups **A** and **B** with small volumes of *Bacillus subtilis*, *Thalassospira sp.*, *Bacillus niabensis* or *Sporosarcina sp.* to give an OD value of 0.08 at 650nm (OD₆₅₀ as described in step 1 above). Methanol was not added to the flasks in Group A, which served as controls. The cultures were incubated for 120 hours, and samples were taken from each flask for optical density (OD) determination throughout the incubation. When OD values were greater than 0.5, samples were diluted with 10% formalin to obtain a more dilute sample to enable the spectrophotometer measure OD accurately.

At the same time as OD measurement was carried out, 0.1 ml of the culture was withdrawn from the flasks and serially diluted with saline to 10^{-2} , 10^{-4} , 10^{-5} and 10^{-6} . 100 μ l of dilutions 10^{-5} and 10^{-6} were plated out on marine agar plates and were incubated at 25°C for 120 hours. A viable colony count was then performed.

BDHAC Degradation Experiment

From the frozen stock in the freezer, strains were streaked out on marine agar plates and were allowed to grow to single colonies. Four universal bottles containing 20ml of marine broth were autoclaved at 121°C before being inoculated by single colonies of the strains of bacteria from the agar plates and incubated at 25°C for 120 hrs. Thereafter, Optical Density was measured. The initial Optical Density value that was required for Step 2 of the experiment was 0.08 in 100ml of growth media, so a measured volume i.e. 0.25ml was taken out of the 20ml culture and was diluted four times with 10% formalin. Optical density of the sample was then following procedures described in Step 1 of the previous section.

Twenty four flasks each with 100ml of marine broth were autoclaved and 0.2ml (0.1mg/ml), 0.5ml (0.25mg/ml), 1ml (0.5mg/ml) and 2ml (1mg/ml) of 5% BDHAC was added to different flasks. 2ml of methanol was added to four flasks, and no additions were

BIODEGRADATION OF BDHAC BY FOUR STRAINS INCUBATED IN MARINE BROTH

made to the final four. The flasks with 2ml of methanol and that without methanol were the controls. The 24 flasks were then inoculated with *Bacillus subtilis*, *Thalassospira sp.*, *Bacillus niabensis* and *Sporosarcina sp.* at a starting OD₆₅₀ of 0.08 resulting in six flasks per bacterium. The flasks were placed on a rotary incubator at 25°C and allowed to shake for 120 hours. Before placing the flasks on the shaker (i.e. at 0 hours), 0.1 ml of the growth media in the flasks was withdrawn and serially diluted with saline to concentrations 10⁻², 10⁻⁴, 10⁻⁵ and 10⁻⁶. 100 µl of the dilutions at 10⁻⁵ and 10⁻⁶ were plated out on marine agar plates. The plates were incubated at 25°C for 120 hours and viable colony counts were performed. In addition, 1ml samples were taken for OD determination followed by the withdrawal of 2 ml samples for extraction with dichloromethane (DCM) and subsequent analysis for residual BDHAC by CDBA. For optical density (OD) measurement, samples with values greater than 0.5 were diluted in 10% formalin and subsequent measured values were multiplied by the dilution factor. The procedures were repeated at 8h, 24h, 48h, 72h and 120h, and were carried out in duplicate.

Analytical Methods

CDBA was used for quantification of residual BDHAC and statistical software (SPSS version 16.0) was used for statistical analysis.

Preparation of Calibration Curve

50mg of BDHAC was dissolved in methanol in a 50ml volumetric flask resulting in 1mg/ml or 1000mg/l standard (**std.1**). 2.0, 1.0, 0.5, 0.25 and 0.1 ml of **std.1** were diluted to 50ml in volumetric flasks with growth medium and the resulting concentrations were 40, 20, 10, 5 and 2mg/l.

Quantification of Residual BDHAC by Colorimetric Dye Binding Assay (CDBA)

Quantification of residual BDHAC was carried out as described in section 2.3.7. 2ml solution was withdrawn from each culture in the different flasks with the bacterial strains at

BIODEGRADATION OF BDHAC BY FOUR STRAINS INCUBATED IN MARINE BROTH

hours 0, 8, 24, 48, 72 and 120 of the degradation experiment and centrifuged to remove cells before extraction with 10ml DCM in a 100ml separating funnel. The 10ml DCM extracts were placed in a 50ml volumetric flask and blown to dryness in gentle stream of nitrogen. Residues were taken up in sea water up to the 50ml mark and subsequently analysed by colorimetric dye binding assay (CDBA) using the same procedures described in Chapter 2 section 2.3.7.

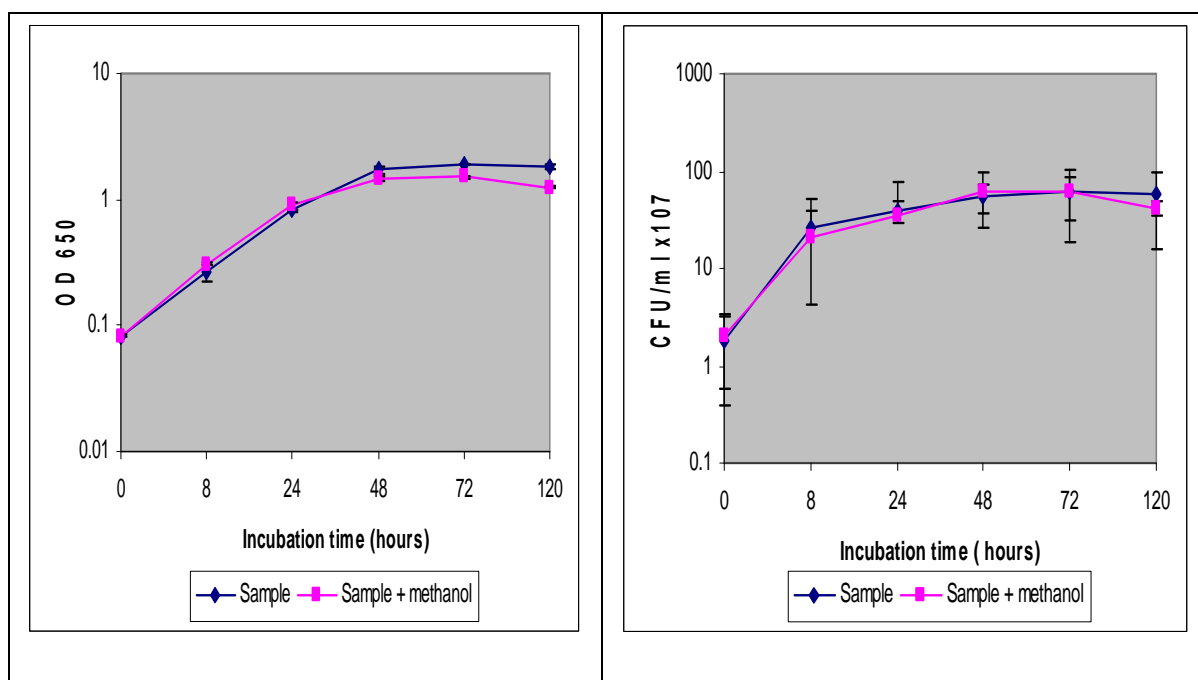
BDHAC Recovery Analysis

To correct losses due to adsorption to glassware, a recovery experiment was carried out to determine the amount of BDHAC adsorbed. Recovery values obtained from this experiment were then used to correct the degradation values obtained from the degradation experiment. For the recovery experiment, four 250ml flasks labelled A, B, C and D, representing four different concentrations of BDHAC, contained 100ml of marine broth. The flasks contained 0.2 ml, 0.5 ml, 1 ml and 2 ml of 5% BDHAC stock standard giving final concentrations of 0.1, 0.25, 0.5 and 1mg/ml. The flasks were left for 120 hours before 2.0 ml of solution in the flasks were withdrawn and used for the recovery experiment. The samples withdrawn were extracted with 10ml of DCM in a 100ml separating funnel and the extracts were placed in 50ml volumetric flasks and were blown to dryness in gentle stream of nitrogen. Residues of BDHAC were taken up in sea water to the 50ml mark and subsequently analysed by colorimetric dye binding assay (CDBA) as described in previous section.

3.16 Results

Determination of the Effect of Methanol on the Growth of Bacteria

Results obtained for the determination of the effect of methanol on the growth of the bacteria are presented in Figures 3.28 – 3.31 and discussed in this section. The volume of inoculum added to the flasks before incubation ranged from 4.5 to 5.5 ml and the initial OD values obtained in the experimental cultures were approximately 0.08 for all the strains.



Error bars represent (Mean \pm 1 S.D); n=2

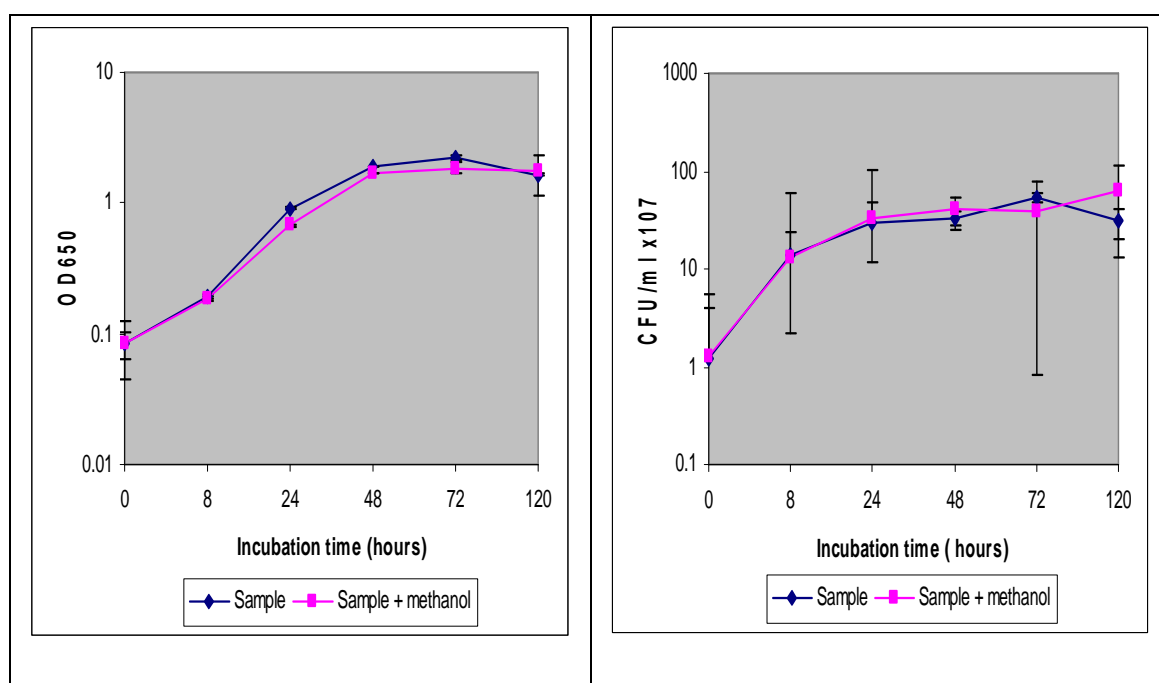
Figure 3.28 Graph Showing OD₆₅₀ and CFU/ml Values for *Bacillus niabensis* in the Presence and Absence of 2 % Methanol.

Growth of *Bacillus niabensis* is shown in Figure 3.28. Growth during the 120 h of incubation was determined by the measurement of optical density (OD₆₅₀) and viable colony counts (CFU/ml). Both growth measurements showed growth of the bacteria in the presence and absence of methanol. Optical Density was shown to increase slightly higher in sample without methanol compared to sample with methanol. Growth levels shown by viable colony counts showed similarity with that obtained from Optical Density measurement. Generally, from the results obtained, it could be deduced that 2ml of methanol in the growth medium did not inhibit the growth of *Bacillus niabensis* in the growth medium.

BIODEGRADATION OF BDHAC BY FOUR STRAINS INCUBATED IN MARINE BROTH

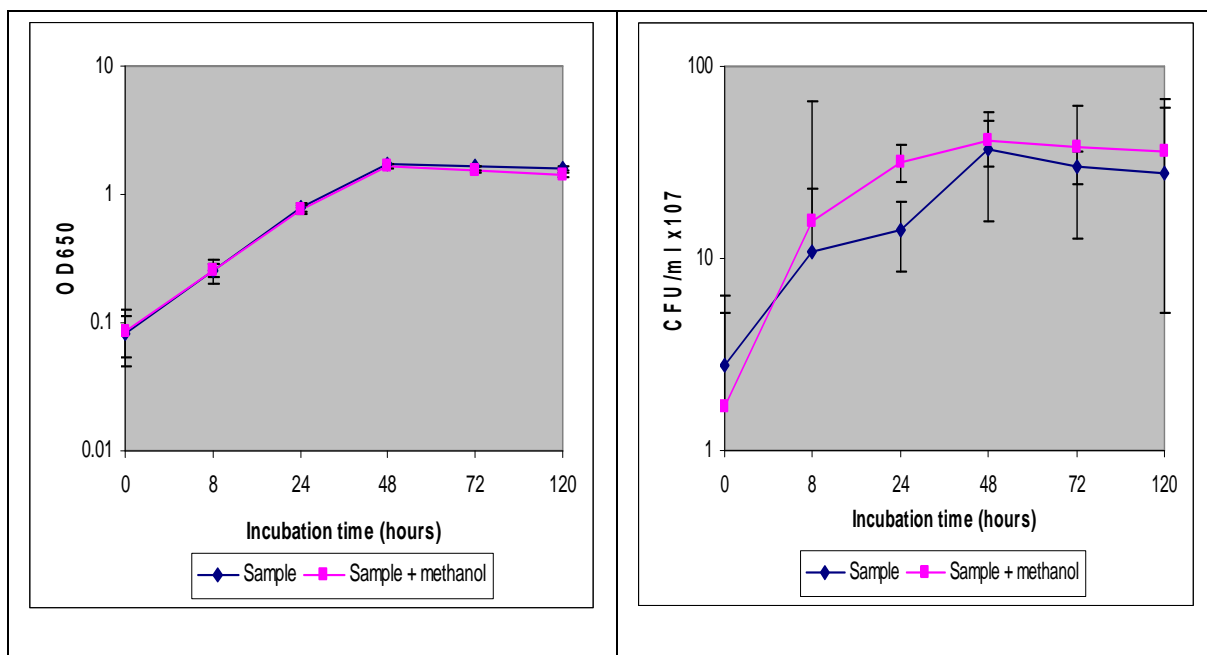
Growth results for *Sporosarcina sp.* are shown in Figure 3.29. Growth increased as the experiment progressed. Growth of the strain in the culture without methanol (OD₆₅₀) increased a bit more than in sample with 2ml of methanol after 48h of incubation. Growth obtained by viable colony counts appeared to decrease in the culture without methanol after 72 hours of incubation.

Based on the data obtained for growth of the strain in both media (i.e. medium with and without methanol), it was deduced that 2% of methanol present in the growth medium, had little or no effect on the growth of *Sporosarcina sp.* in 120 h of incubation..



Error bars represent (Mean \pm 1 S.D); n=2

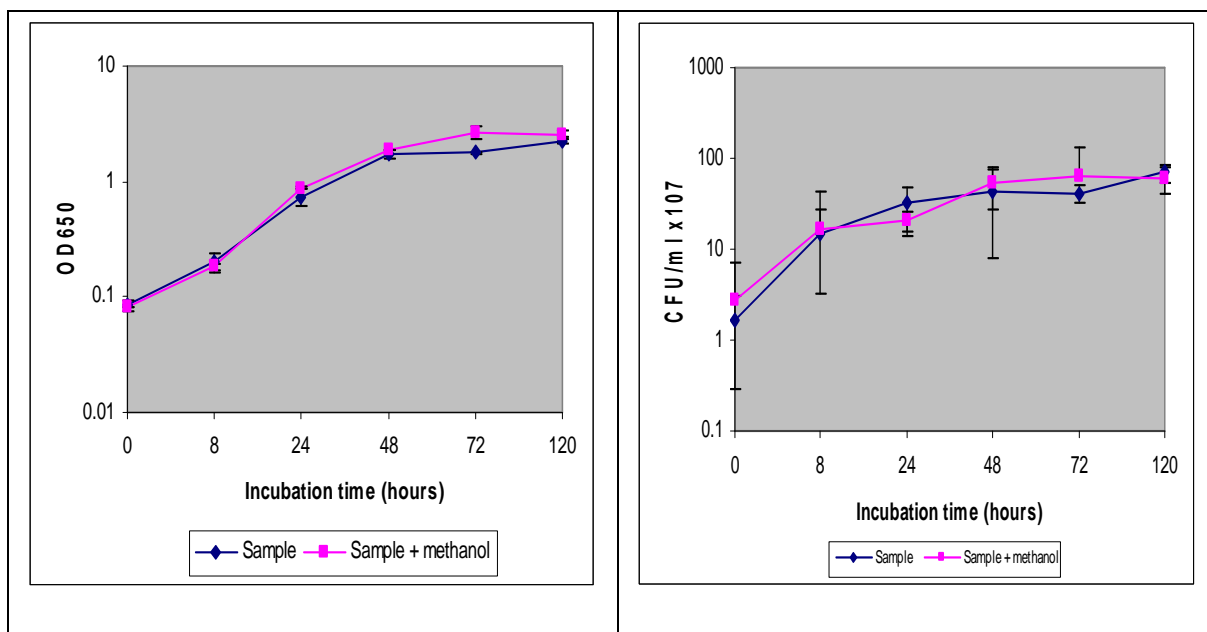
Figure 3.29 Graph Showing OD₆₅₀ and CFU/ml Values for *Sporosarcina sp.* in the Presence and Absence of 2 % Methanol.



Error bars represent (Mean \pm 1 S.D); n=2

Figure 3.30 Graph Showing OD₆₅₀ and CFU/ml Values for *Bacillus subtilis* in the Presence and Absence of 2 % Methanol.

Figure 3.30 show OD₆₅₀ and CFU/ml values for *Bacillus subtilis* in growth media with and without methanol. Growth (OD₆₅₀) of the strain was shown to increase steadily for sample without methanol and sample with methanol during the first 48 h of incubation and then levelled off. A similar trend was observed for growth followed by measuring CFU/ml. The decrease in growth was observed for both samples with and without methanol showing that methanol was not responsible for the decrease. Generally, the results obtained revealed that methanol was not toxic to *Bacillus subtilis* when it was present in the growth medium.



Error bars represent (Mean \pm 1 S.D); n=2

Figure 3.31 Graph Showing OD₆₅₀ and CFU/ml Values for *Thalassospira sp.* in the Presence and Absence of 2 % Methanol.

Growth results for *Thalassospira sp.* are shown in Figure 3.31. Growth increased for sample with and without methanol as observed from results obtained from OD₆₅₀ and CFU/ml measurements. Growth of the strain in the presence of methanol was slightly higher than that without methanol. The growth pattern showed a similar trend for both media i.e. steady growth throughout the incubation period. Generally, results obtained for growth of the strain in both growth media showed that methanol did not inhibit its growth during 120 h of incubation.

Relationship between OD and CFU/ml

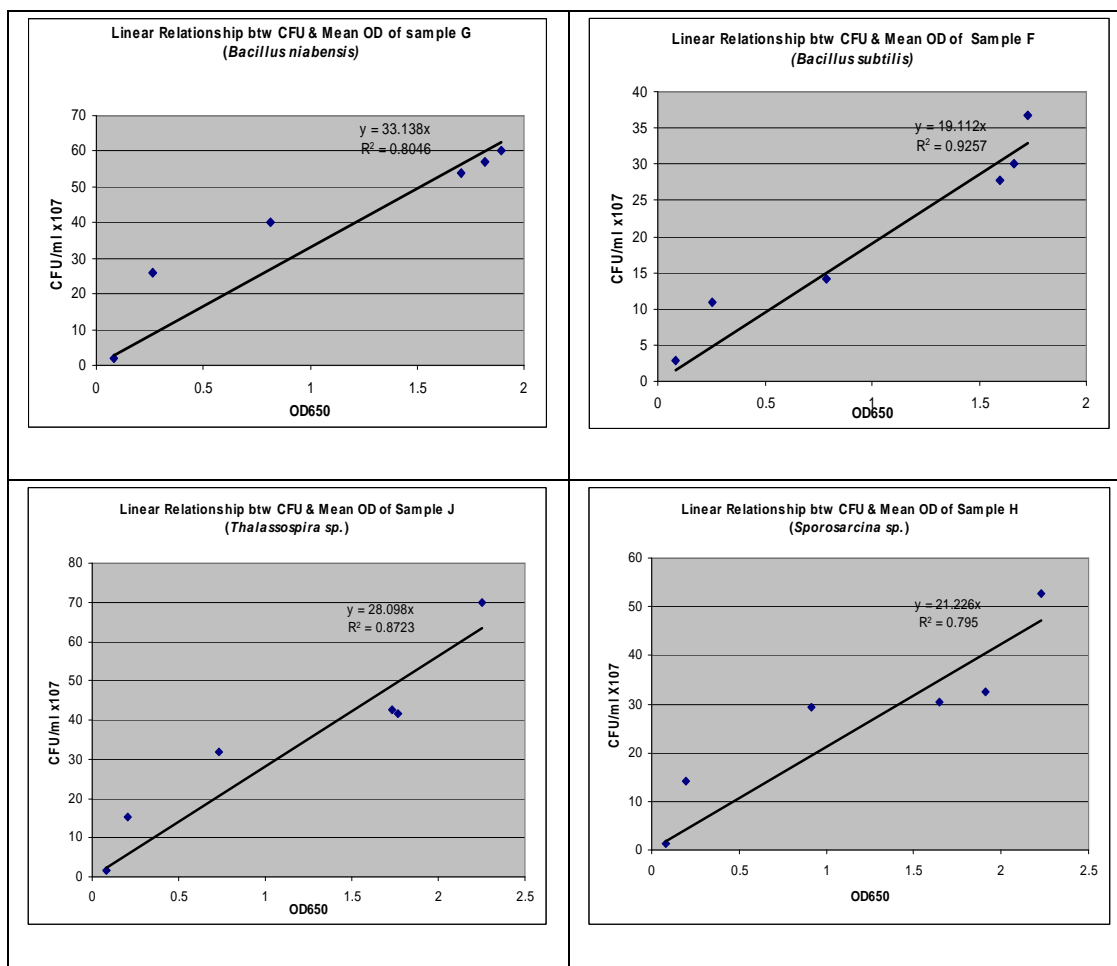


Figure 3.32 Relationship of Colony Forming Unit (CFU/ml x10⁷) and Optical Density (OD₆₅₀) for the different Strains of Bacteria.

Plots of CFU/ml against OD₆₅₀ for all the strains in medium without methanol are shown in Figure 3.32. The plots showed a linear relationship between OD₆₅₀ and CFU/ml values obtained from the growth investigation. A close relationship between OD and CFU was observed for *Bacillus subtilis* ($R^2=0.9257$). This was closely followed by that for OD and CFU for *Thalassospira sp.* ($R^2=0.8723$). The positive relationship between OD₆₅₀ and CFU validates the use of OD as a measure of growth of the bacteria.

BIODEGRADATION OF BDHAC BY FOUR STRAINS INCUBATED IN MARINE BROTH

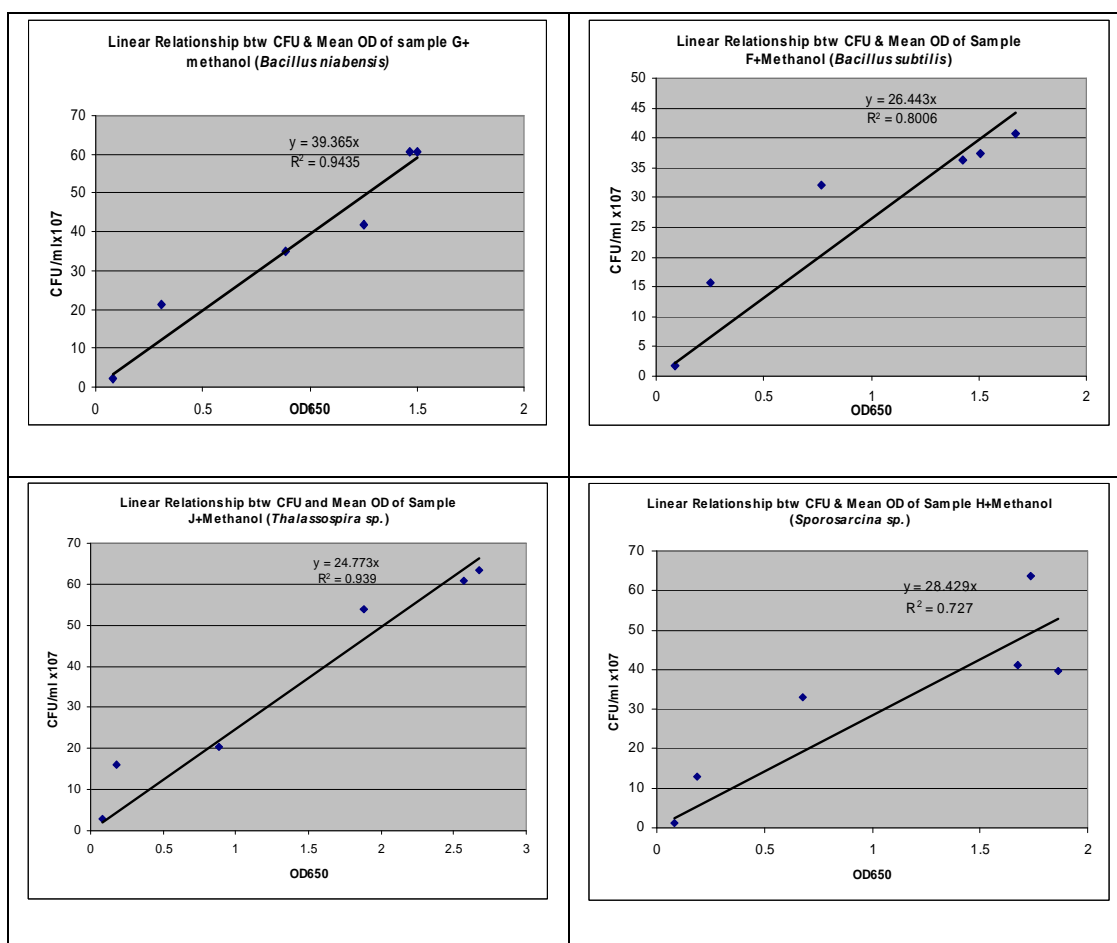


Figure 3.33 Relationship of Colony Forming Unit (CFU/ml x10⁷) and Optical Density (OD₆₅₀) for the different Strains of Bacteria in the presence of 2ml of Methanol

The same was found to be true for cultures grown in the presence of methanol. Figure 3.33 illustrates the relationship between OD₆₅₀ and CFU/ml values from these experiments. The linear plots revealed particularly close relationship between OD and CFU/ml values for *Bacillus niabensis* and *Thalassospira sp.* ($R^2=0.9435$ and 0.939) respectively. From the results obtained from the experiments comparing growth in the presence and absence of methanol, it was generally deduced that 2 % (v/v) methanol present in the growth medium did not inhibit the growth of any of the four strains of bacteria. Based on this evidence, BDHAC for the degradation experiments was prepared at a concentration such that not more than 2 ml of methanol would be added to 100 ml volumes of culture.

BDHAC Degradation Experiment

An investigation was carried out on the biodegradation of BDHAC by *Bacillus subtilis*, *Bacillus niabensis*, *Sporosarcina sp.* and *Thalassospira sp.* cultivated in marine broth. BDHAC was present at concentrations of 0.1, 0.25, 0.5 and 1mg/ml and was a potential source of carbon and energy for the bacteria. In addition controls without BDHAC and with 2 % methanol were set up and the experiment was allowed to run for 120 hours.

The volume of inoculums added to the flasks before incubation was around 4.5 ml, and the initial OD values in the experimental cultures were approximately 0.08 for all the strains.

Bacillus niabensis

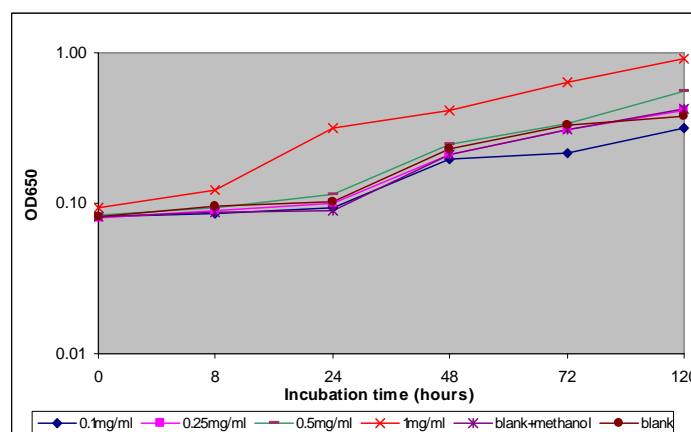


Figure 3.34 Growth of *Bacillus niabensis* (OD650) at Different Concentrations of BDHAC During 120 hours of Incubation.

Table 1 (Appendix E.2) and Figure 3.34 show growth of *Bacillus niabensis* in marine broth with different concentrations of BDHAC and in the control cultures over 120 h of incubation. The highest level of growth was observed at the highest concentration of BDHAC, 1mg/ml. A similar growth pattern was observed for cultures containing 0.5, 0.25 mg/ml BDHAC and the two controls.

Bacillus subtilis

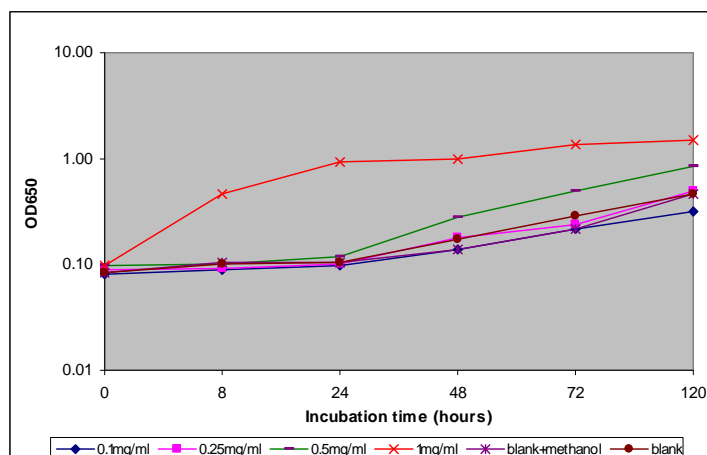


Figure 3.35 Growth of *Bacillus subtilis* (OD650) at Different Concentrations of BDHAC in 120 Hours of Incubation.

Table 2 (Appendix E.2) and Figure 3.35 show the growth of *Bacillus subtilis* in growth medium with different concentrations of BDHAC and the controls. The highest growth level was observed for BDHAC concentration of 1mg/ml. Growth observed in the presence of 0.5 mg/ml BDHAC was also higher than in the presence of 0.1 and 0.25 mg/ml and the controls, which showed a similar level of growth throughout the experiment.

Sporosarcina sp.

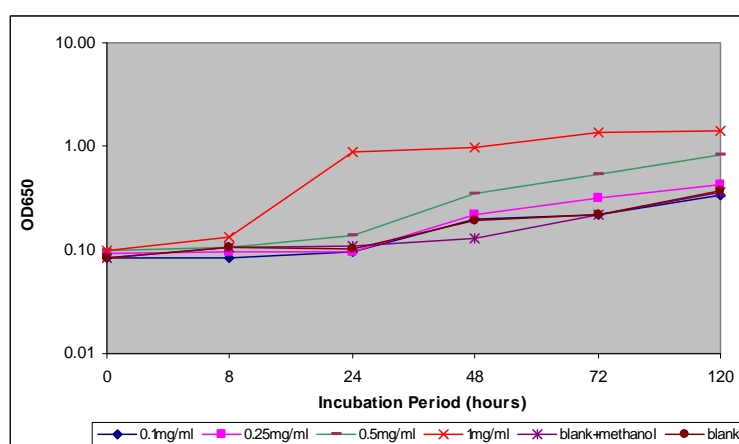


Figure 3.36 Growth of *Sporosarcina sp.* (OD650) at Different Concentrations of BDHAC During 120 hours of Incubation

BIODEGRADATION OF BDHAC BY FOUR STRAINS INCUBATED IN MARINE BROTH

Sporosarcina sp. grew in the presence of concentrations of BDHAC and in the two control cultures as shown in Table 3 (Appendix E.2) and Figure 3.36. Growth increased with incubation time and the highest level of growth was observed for a BDHAC concentration of 1mg/ml. This was followed by 0.5 mg/ml. Cultures containing 0.1 and 0.25 mg/ml BDHAC showed similar growth to the control cultures.

Thalassospira sp.

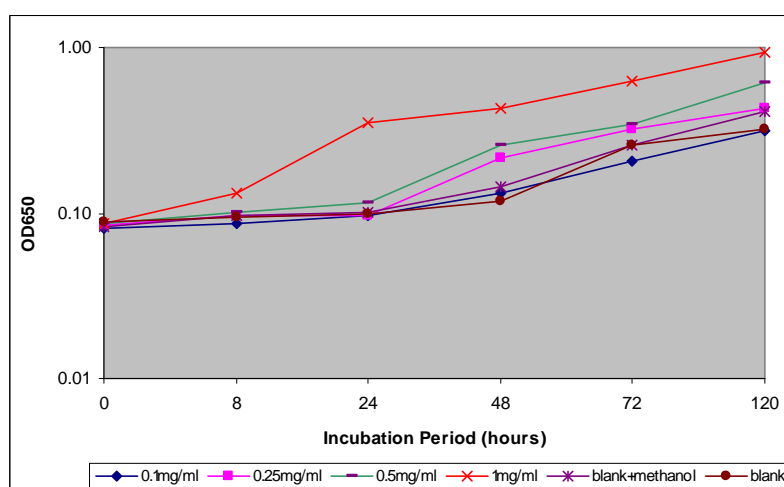


Figure 3.37 Growth of *Thalassospira sp.* (OD₆₅₀) at Different Concentrations of BDHAC During 120 hours of Incubation.

Table 4 (Appendix E.2) and Figure 3.37 show the growth of *Thalassospira sp.* in growth medium with different concentrations of BDHAC and in the control cultures (blank with and without methanol). A good level of growth was observed for BDHAC concentration of 1mg/ml. Growth of the strain at 0.5 and 0.25 mg/ml were slightly higher than observed at 0.1mg/ml and the control cultures. Growth of *Thalassospira sp.* in 120 h of incubation showed that up to 1mg/ml BDHAC was not toxic to the strain. Generally, the growth results were consistent in that high concentration of up to 1mg/ml BDHAC resulted in more growth. This indicated that the four strains were utilising this substance as a source of carbon and energy. However, the strains of bacteria did not grow as well as they did in the

BIODEGRADATION OF BDHAC BY FOUR STRAINS INCUBATED IN MARINE BROTH

experiments described in section 3.15. This can be attributed to the fact bacteria may go into a prolonged lag phase during growth.

Preparation of Calibration curve for the Quantification of Residual BDHAC by CDBA.

Results obtained from a calibration curve prepared are as summarised in Table 3.13. From the analysis, absorbance values were recorded at a wavelength of 450nm and a calibration curve (Figure 3.38) was plotted. The curve showed linearity ($R^2 = 0.972$) signifying a good relationship. The equation of the line generated from the calibration curve was used in calculating the concentrations of BDHAC obtained through analysis by the Colorimetric Dye Binding Assay.

Table 3.13 Concentration, Volume and Absorbance of Calibration Standards

| Concentration of BDHAC (mg/ml) | Absorbance |
|--------------------------------|------------|
| 20 | 2.863 |
| 10 | 1.825 |
| 5 | 0.931 |
| 2 | 0.304 |

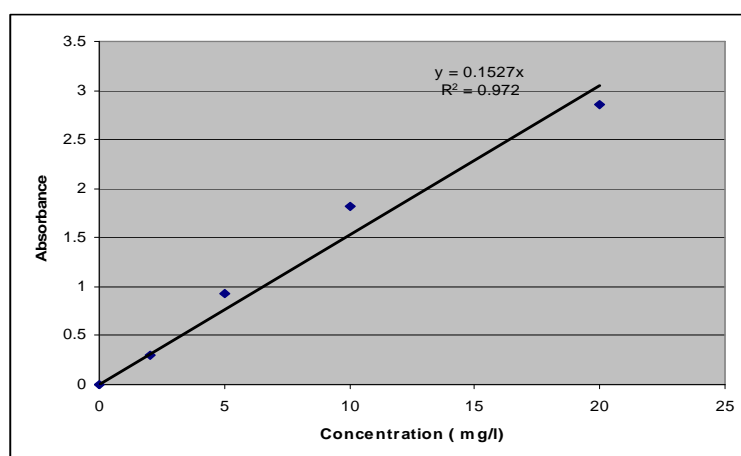
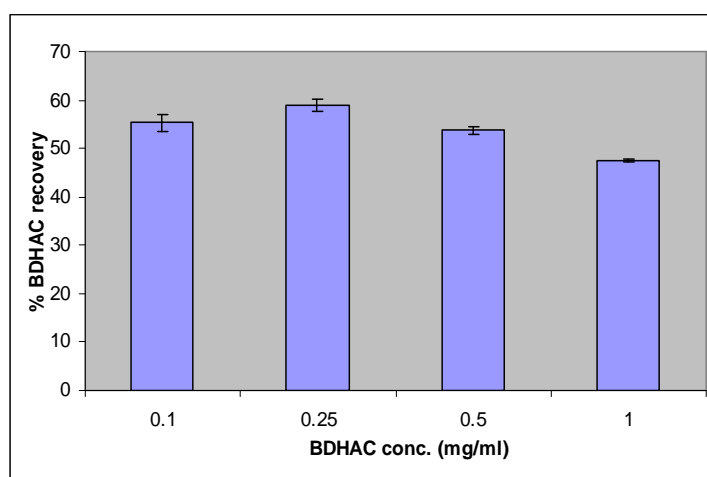


Figure 3.38 Calibration Curve for CDBA

BDHAC Recovery Analysis

A recovery experiment was carried out to determine the actual amount of BDHAC lost due to biodegradation. This was carried out following the procedures described in Section C. From the absorbance values obtained from the recovery experiment, simple calculations (Appendix A.4) were used to quantify the amount of BDHAC recovered from solution.



Error bars represent (Mean \pm 1 S.D); n=2

Figure 3.39 Percent Recovery of BDHAC at Different Concentrations

Figure 3.39 show percent recovery values for different concentrations of BDHAC. From the results, it was observed that the highest percent recovery value 58.88% was obtained for 0.25 mg/ml BDHAC. This was not expected as recovery experiments in Sections A and B showed high recoveries at high concentrations. However, the recoveries were reasonable and showed that BDHAC can be recovered from its solution during degradation experiments.

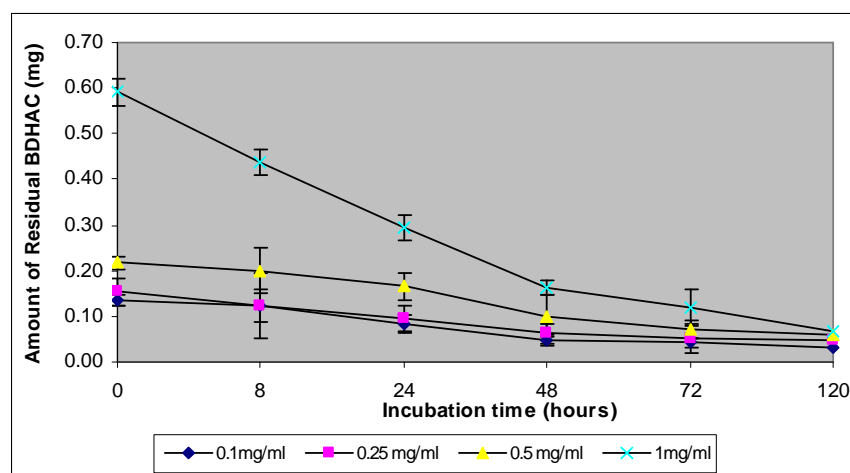
Quantification of residual BDHAC by CDBA

After 120 hours of the biodegradation experiment with BDHAC concentrations of 0.1, 0.25, 0.5 and 1mg/ml, the amount of residual BDHAC was quantified to check for losses due to

BIODEGRADATION OF BDHAC BY FOUR STRAINS INCUBATED IN MARINE BROTH

biodegradation. This was performed using the Colorimetric Dye Binding Assay (CDBA) and the results obtained expressed as amount of residual BDHAC are presented and discussed in this section. Sample calculations for degradation of BDHAC are shown in Appendix E.1

Bacillus niabensis

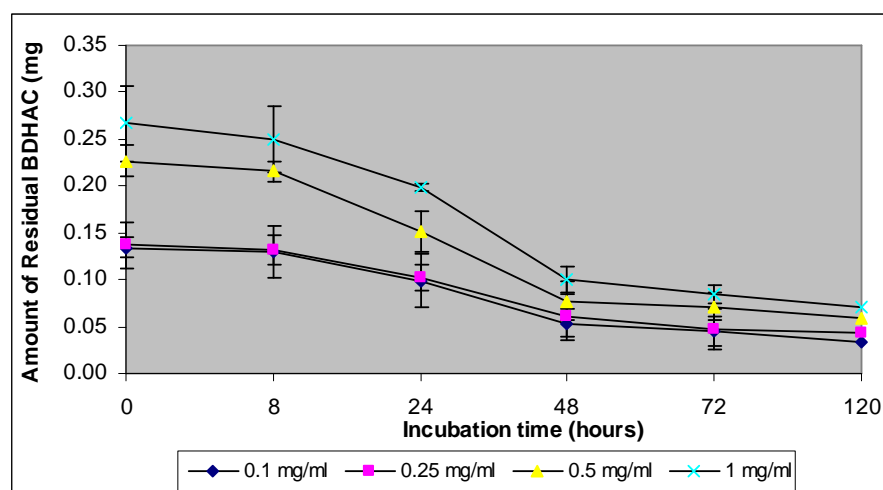


Error bars represent (Mean \pm 1 S.D); n=2

Figure 3.40 Residual Amount of BDHAC from cultures of *Bacillus niabensis* Incubated for 120 hours.

The residual amount of BDHAC recovered from growth media during growth of *Bacillus niabensis* is presented in Table 6 (Appendix E.2) and Figure 3.40. It can be observed that there was a decrease in the amount of BDHAC recovered during the course of the experiment. It was observed that the results from the degradation experiment agreed with that obtained from the growth experiments i.e. more growth resulted in more disappearance of BDHAC from the growth medium. Also cultures with no BDHAC added did not show presence of the substance when assayed. Therefore *Bacillus niabensis* could degrade BDHAC during growth when it is present at a concentration of up to 1 mg/ml.

Bacillus subtilis.

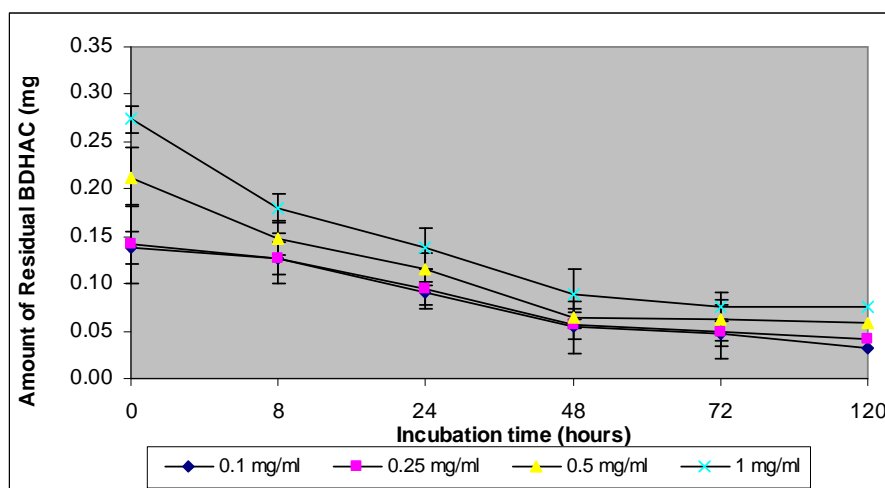


Error bars represent (Mean \pm 1 S.D.); n=2

Figure 3.41 Residual Amount of BDHAC from Cultures of *Bacillus subtilis* Incubated for 120 hours.

Table 8 (Appendix E.2) and Figure 3.41 show the BDHAC recovered from cultures of *Bacillus subtilis* during 120 hours of incubation. The amount of BDHAC amounts was shown to decrease with incubation time. This was in agreement with the growth of the bacteria that showed a high level of growth of *Bacillus subtilis* in the presence of 0.5 mg/ml or 1 mg/ml BDHAC. These findings indicate the utilization of the different concentrations of BDHAC by *Bacillus subtilis*.

Sporosarcina sp.

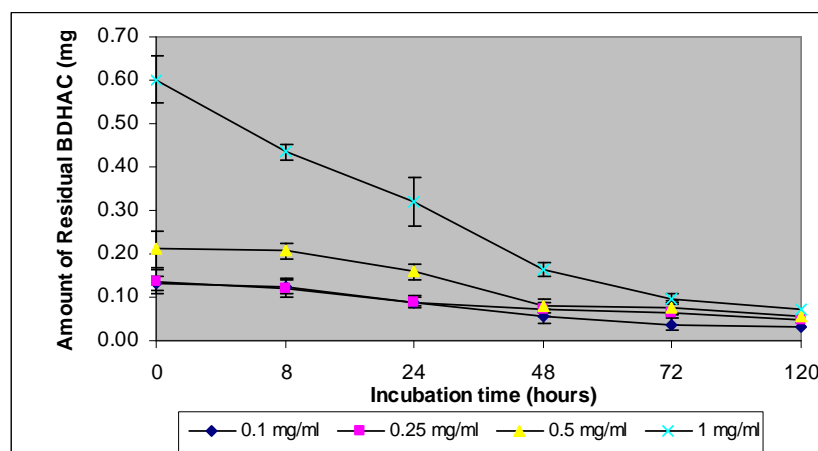


Error bars represent (Mean \pm 1 S.D); n=2

Figure 3.42 Residual Amount of BDHAC from Cultures of *Sporosarcina sp.* Incubated for 120 hours.

Table 10 (Appendix E.2) and Figure 3.42 presents the decrease in the amount of BDHAC during the growth of *Sporosarcina sp.* over a period of 120 h. Amounts of BDHAC were shown to decrease with incubation time and the decrease was large in samples with 1 and 0.5 mg/ml BDHAC. As for the other bacteria, it was deduced that the disappearance of BDHAC was due to biodegradation. Generally, there was decrease in the amount of BDHAC in growth medium from the start of the experiment. This finding suggests that *Sporosarcina sp.* had utilised BDHAC during growth.

Thalassospira sp.



Error bars represent (Mean \pm 1 S.D); n=2

Figure 3.43 Residual Amount of BDHAC from Cultures of *Thalassospira sp.* Incubated for 120 hours.

Table 12 (Appendix E.2) and Figure 3.43 show the residual amount of BDHAC recovered from the medium during growth of *Thalassospira sp.* Amounts of BDHAC in the growth medium were shown to decrease with increase in incubation time. Results from the degradation experiment showed a general decrease in the amounts of BDHAC recovered for the four different concentrations over time. The results obtained indicated that *Thalassospira sp.* could utilise BDHAC at concentrations of 1 mg/ml when it was present in the growth medium.

Generally, there was consistency in the results obtained from the degradation experiments for the different strains of bacteria. The different concentrations of BDHAC in the growth medium decreased with increase in incubation time. The loss in the amount of BDHAC observed during the start of the experiment (0 hr) may have been as a result of the centrifugation of BDHAC cultures prior to extraction with DCM, as losses due to adsorption were carefully corrected for by performing a separate recovery experiment. BDHAC that was bound to cell material in the culture solution may have been removed by the centrifugation process, thereby reducing the amount of BDHAC. Also the surface charge of the strains in solution was not measured.

3.17 Discussion

Bacillus niabensis, *Sporosarcina sp.*, *Bacillus subtilis* and *Thalassosopira sp.* were cultivated in marine broth with 0.1, 0.25, 0.50 and 1.0 mg/ml benzyldimethylhexadecylammonium chloride (BDHAC). The strains grew well in media with higher concentrations of BDHAC compared to the controls with 2% of methanol and that with no BDHAC. BDHAC concentrations of 0.5 and 1.0 mg/ml in particular enhanced the growth of the different strains, and in addition, the strains degraded these concentrations of the BDHAC to a large extent. Biodegradation of BDHAC at low concentrations by Gram-positive bacteria (*Bacillus niabensis* and *Bacillus subtilis*) supports the hypothesis that regular exposure of some Gram-positive bacteria to QACs results in adaptation and resistance to these chemicals (Sundheim et al., 1992). Also, Gram-negative bacteria such as *Psuedomonas sp.* have been reported to adapt to sanitisers used in food processing industries (Langsrud and Sundheim 1997 and Langsrud et al., 2003). *Bacillus niabensis*, *Sporosarcina sp.*, *Bacillus subtilis* and *Thalassosopira* can degrade BDHAC and growth levels were shown to increase with an increase in concentration of BDHAC. Results from CDBA analysis and the growth experiments suggest that the strains could grow better when cultivated in marine broth than in minimal salts, while using BDHAC as a carbon and energy source. A detailed discussion of the findings from the entire BDHAC degradation studies can be seen in Chapter 5.

3.18 Conclusion

This study provides evidence that bacterial strains cultivated in marine broth degraded BDHAC when it was present at lower concentrations than reported in Section B. Analysis of breakdown products generated from this study was not feasible due to the breakdown of the mass spectrometer. The study has confirmed that pure cultures of microorganisms from marine sediments could utilize BDHAC as a carbon and energy source when the QAC was present at concentrations in the range of 0.05-4 mg/ml. It also confirmed the observation by Patrauchan and Oriel, (2003) that the alkyl chains in a QAC with a benzyl ring can be degraded by bacteria.

CHAPTER 4

IDENTIFICATION OF PRODUCTS FROM BACTERIAL DEGRADATION OF BDHAC

4.1 Introduction

Biodegradation of quaternary ammonium compounds by micro-organisms has been identified in the OECD/EEC screening test (Larson and Perry 1981, Masuda et.al, 1976). However, the results from these simple biodegradation tests do not exclude the formation of recalcitrant intermediates. Degradation pathways of organic compounds are vital proof of total mineralization of the organic compounds.

Biodegradation of QACs will result in the formation of metabolic intermediates (van Ginkel et al., 1992). It was important to investigate the potential metabolites from BDHAC metabolism, since information obtained will give an understanding of the pathway through which the strains of bacteria metabolised it.

ESI-MS/MS was used to study possible metabolites of BDHAC degradation, including N-benzylmethylamine (BMAM), benzoic Acid (BA), and benzaldehyde (BAH). These metabolites were selected based on the findings of Patrauchan and Oriel (2003). These authors investigated the degradation of benzyldimethylalkylammonium chloride (BAC) by *Aeromonas hydrophila* sp. K and reported that the biodegradation of a benzyl-containing quaternary ammonium compound such as BAC yielded benzyldimethylamine, benzylmethylamine, benzylamine, benzaldehyde and benzoic acid.

4.2 Aim of the Study

The aim of this study is to establish clearly the biodegradation of BDHAC by identifying the products of its transformation by bacteria isolated from marine sediment.

4.3 Materials and Methods

N,N-dimethylbenzylamine (BDAM), N-benzylmethylamine (BMAM), benzoic Acid (BA), and benzaldehyde (BAH) were purchased from Sigma Aldrich, UK Ltd.

4.3.1 Preparation and analysis of standards

For the identification of metabolites from BDHAC degradation, 3.80mg N, N-dimethylbenzylamine was added to a 10 ml volumetric flask and made up to the mark with 90:10 methanol: water. 1ml of this stock standard was diluted 10 times and the resultant solution was analysed by ESI-MS/MS to obtain a mass spectrum for the compound. The spectrum indicated both the parent ion at m/z 136 and fragment ion at m/z 91, corresponding to C₇H₇ (the benzyl ring). The abbreviation m/z is used to denote the quantity formed by dividing the mass number of an ion by its charge number. The parent/daughter ion transition (136→91) was subsequently selected for the confirmatory analysis.

50 ml culture samples from the last day of the BDHAC degradation experiment were centrifuged to remove cells and subsequently extracted with 20ml of DCM, and the extracts were divided into two equal portions. One portion (10 ml) of the extract was blown to dryness in a gentle stream of N₂ and re-dissolved in 90:10 methanol: water. 1ml of the resultant solution was diluted to 10 times the volume and analysed by ESI-MS/MS to check for a peak at a transition of 136→91.

4.4 Results

N, N-dimethylbenzylamine was observed from preliminary analysis as a product in the surfactant transformation. This suggests that the initial attack on BDHAC molecules is a central fission of the C-alkyl-N bond. This finding supports the view that C-alkyl-N fission of alkyl compounds by micro-organisms is a general strategy to gain access to the alkyl chains (van Ginkel, 1995; Patrauchan and Oriel, 2003).

IDENTIFICATION OF PRODUCTS FROM BACTERIAL DEGRADATION

Analysis by electrospray ionisation mass spectrometric in the multiple reactions monitoring mode (MRM) showed a peak at a transition of 136.0→91.0 corresponding to benzyldimethylamine (BDAM) when samples derived from cultures of the strains were analysed for possible metabolites after the degradation and quantification processes. BDAM was formed as an intermediate of BDHAC degradation by *Thalassospira sp.*, *Bacillus subtilis*, *Sporosarcina sp.* and *Bacillus niabensis*. Figure 4.14b illustrates the formation of BDAM after degradation of BDHAC by *Bacillus niabensis*. A similar observation was made for *Thalassospira sp.*, *Bacillus subtilis*, *Sporosarcina sp.* but chromatograms could not be retrieved due to the breakdown of the analytical equipment.

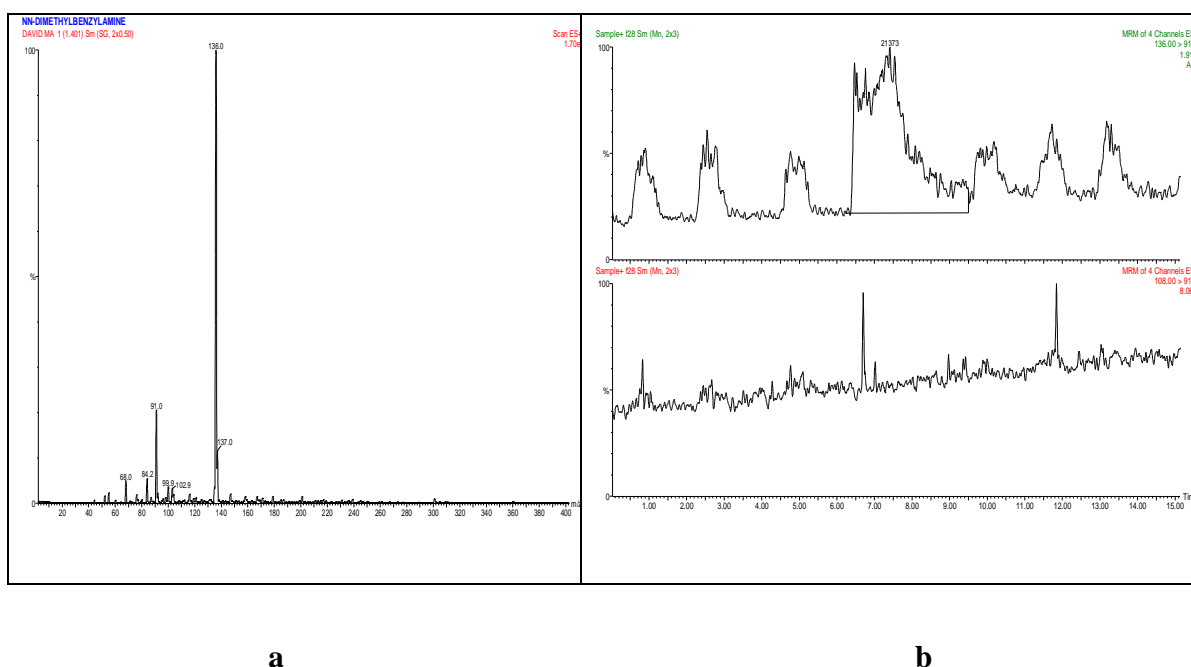


Figure 4.1 ESI-MS Spectra and Chromatogram Showing Pure Standard of Benzyldimethylamine and that Formed after Biodegradation of BDHAC by the Strains of Bacteria.

The peak shown on Figure 4.1a is at the transition of 136.0→ 91.0 representing the pure standard of benzyldimethylamine, while Figure 4.1b shows the formation of N, N-dimethylbenzylamine as an intermediate of BDHAC catabolism (i.e. the fourth broad peak).

IDENTIFICATION OF PRODUCTS FROM BACTERIAL DEGRADATION

The smaller peaks shown on Figure 4.1**b** represent 90:10 methanol: water that was injected in replicate as blank solvent before and after sample injection to check for carry -over of sample in each case. Each peak on chromatogram **b** represents a new injection. Figure 4.2 show a possible degradation pathway for BDHAC transformation. The proposed pathway for the surfactant degradation was via a central fission of the C-alkyl-N bond.

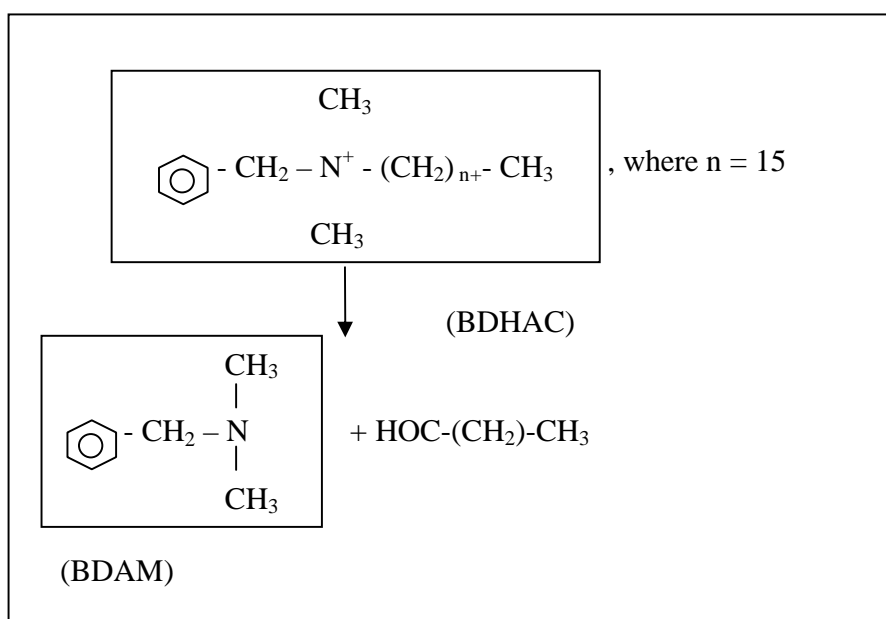
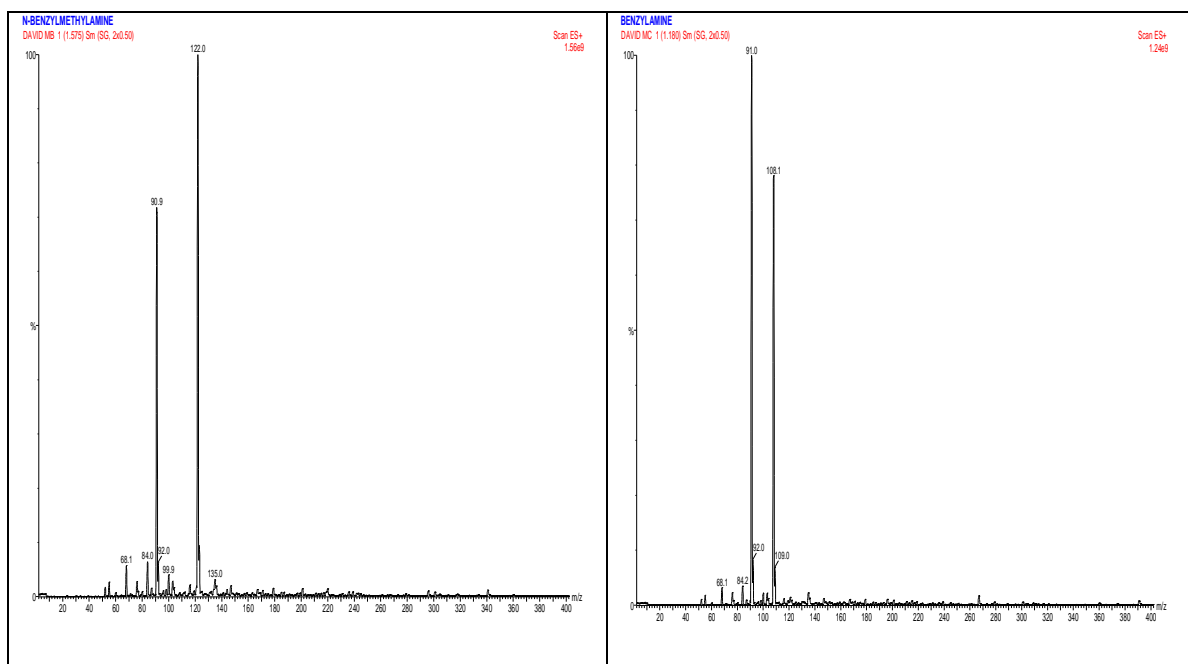


Figure 4.2 Proposed Degradation Pathway of BDHAC Showing the Formation of Benzyldimethylamine (BDAM).

Pure standards of N-Benzylmethylaniline, Benzylamine, Benzaldehyde and Benzoic acid were prepared following the same procedures described for N, N-dimethylbenzylamine, and analysed by ESI-MS to obtain mass spectra for the different compounds. The spectra are shown in Figures 4.3 (c, d) and 4.4 (e, f). Results from the analysis did not show a peak at the selected wavelengths. It was not clear if potential metabolites could not be detected by the ESI-MS technique or were not present as products of BDHAC transformation.

IDENTIFICATION OF PRODUCTS FROM BACTERIAL DEGRADATION

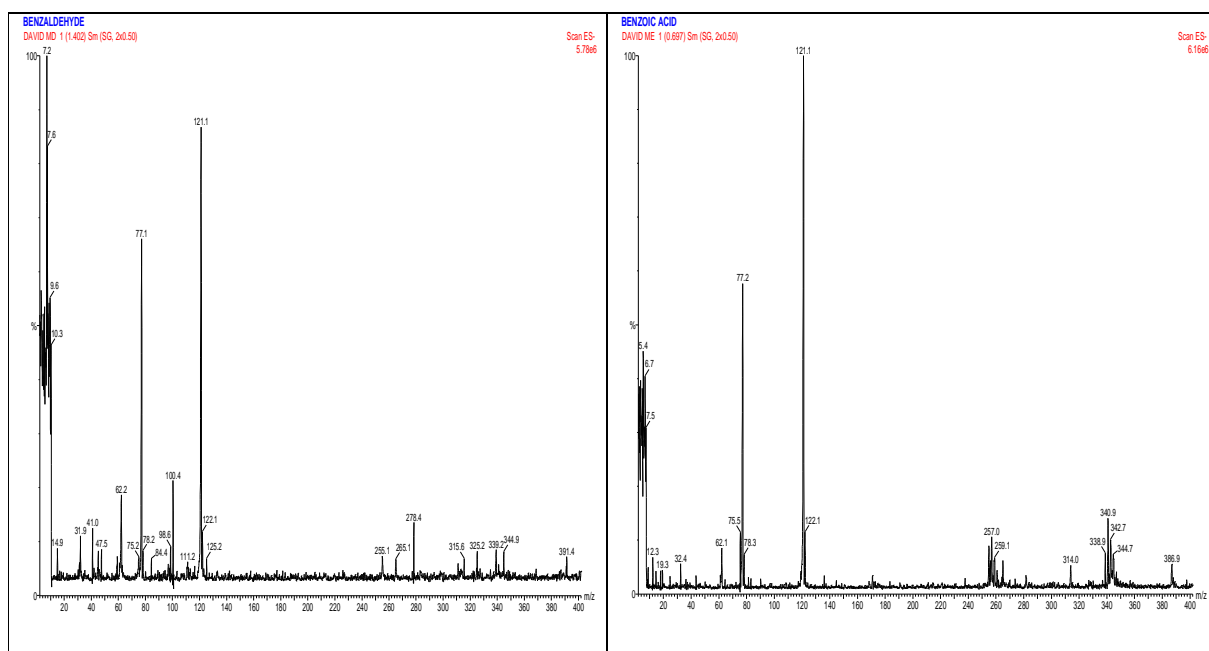


c

d

Figure 4.3 ESI-MS Spectra Showing Pure Standards of (a) N-Benzylmethylamine and (b) Benzylamine that were also Tested.

Figure 4.3c indicated both the parent ion at m/z 122.10 and fragment ion at m/z 91, while Figure 4.3d indicated both the parent ion at m/z 107.15 and fragment ion at m/z 91. The parent/daughter ion transition (122.10→91) for N-Benzylmethylamine and (107.15→91) for Benzylamine was subsequently selected for sample analysis.



e

f

Figure 4.4 ESI-MS Spectra Showing Pure Standards of (e) Benzaldehyde and (f) Benzoic Acid that were also Tested.

Figure 4.4e indicated both the parent ion at m/z 108 and fragment ion at m/z 91, while Figure 4.4f indicated both the parent ion at m/z 122.10 and fragment ion at m/z 77.20. The parent/daughter ion transition ($108 \rightarrow 91$) for Benzaldehyde and ($122.10 \rightarrow 77.20$) for Benzoic acid was subsequently selected sample for analysis.

4.5 Discussion

The biodegradation of QACs by bacteria is still poorly understood. The elimination of pollutants and wastes from the environment requires an understanding of the relative importance of different pathways utilised by microorganisms such as bacteria in the transformation of these chemicals (Lovley, 2003).

Generally, the basic biodegradation steps that have been reported by researchers, includes the initial fission of the carbon-nitrogen bonds, leading to the formation of a tertiary amine and a residual alkanal (Cain, 1976). Other possible mechanisms of QAC degradation by bacteria reported include N-demethylation and N-dealkylation. (Cain, 1976), predicted that the long chains of QACs were susceptible to ω -oxidation at the terminal methyl position. This mechanism of biodegradation in which an attack is initiated at the end of the alkyl chain has also been reported by Dean-Raymond and Alexander (1977). They observed that during the biodegradation of decyltrimethylammonium chloride by a xanthomonad, 9-carboxynonyl- and 7-carboxyheptytrimethylammonium salts were formed as a result of ω -oxidation of the decyl group.

C-N fission (cleavage of the alkyl chain) was proposed by van Ginkel (1992) in a study on the biodegradation of hexadecyltrimethylammonium chloride by *Pseudomonas strain* B1. The bacterium was shown to grow on hexadecanol and hexadecanoate formed as the intermediates. This result was also in line with that of Hampton and Zatman, (1973) that studied the metabolism of tetramethylammonium chloride by *Pseudomonas sp* 5H2. The authors observed that tetramethylammonium chloride was completely degraded and that the oxidation of QAC proceeded by the splitting of the C-N bond, resulting in methanal and trimethylamine. Nishiyama et al., (1995) reported that Alkyltrimethylammonium salts were degraded by N-demethylation and N-dealkylation. The intermediates formed i.e. TMA, alkyldimethylammonium salt and betaine were further degraded by N-demethylation and N-dealkylation processes.

Also, Nishiyama and Nishihara (2002) in their study on the biodegradation of dodecyltrimethylammonium bromide (DTAB) by *P.fluorescens*, proposed that the pathway of the QAC biodegradation was by N-dealkylation to yield trimethyl amine (TMA). TMA was later transformed by N-demethylation to dimethyl amine (DMA). In the biodegradation of didecyldimethylammonium chloride (DDAC) by *Pseudomonas fluorescens* TN4, Nishihara et al., (2000) also suggested that two N-dealkylation steps were involved in the degradation of DDAC.

During studies on the adaptation of *pseudomonas sp.* strain 7-6 to quaternary ammonium compounds, Takenaka et al. (2007) reported that degradation was via dual pathways and proposed possible pathways for DTAC metabolism. DTAC was first

converted to lauric acid via *n*-dodecanal with the release of trimethylamine while in pathway 2; DTAC was converted to lauric acid via *n*-dodecyldimethylamine. Patrauchan and Oriel, (2003) also reported that benzyldimethylalkylammonium chloride was metabolised by C-alkyl-N bond fission to yield benzyldimethylamine (BDAM) followed by the formation of benzylamine (BAM). The BDAM and BAM were subsequently utilized by the bacteria. The pathway proposed for the degradation of BDHAC by bacteria isolated from marine sediment was in line with those proposed for the degradation of benzyldimethylalkylammonium chloride by Patrauchan and Oriel, (2003) and others authors already mentioned. Patrauchan and Oriel, (2003), suggested that C-benzyl-N bond fission was not feasible.

Microorganisms are able to transform pollutants through the production of enzymes, chelating agents, storage bodies and cell surface agents. Organic species such as quaternary ammonium compounds are targeted substrates (Kastner et al., 1999). Microbial degradation involves the breakdown of organic compounds either through biotransformation into less complex metabolites or through mineralization into inorganic minerals, H₂O, CO₂ (aerobic) or CH₄ (anaerobic). Bacteria have been extensively studied for their ability to degrade a range of environmental pollutants. The biochemical pathways required for the transformation stages are often specific for target environmental contaminants, converting them to metabolites which can be assimilated into more ubiquitous bacterial pathways (Parales et al. 2002). However, the inability of microbes to mineralise contaminants but rather transform them partially means that these organisms require other substrate to support their growth. In such cases, the contaminants are transformed by cometabolism.

The mechanisms for aerobic degradation of nitrogen-containing synthetic contaminants such as QACs have not been completely elucidated (Parales et al. 2002). Catalytic reaction types include deaminations, N-dealkylations and nitroreductions. In all these mechanisms, there is involvement of monooxygenases, dioxygenases, nitroreductases and esterases in the first stages of breakdown. Mono- and/or dioxygenases participate in the initial stages of reactions in aerobic bacterial breakdown of nitroaromatics such as QACs (Wackett and Hershberger, 2001).

Reactions catalysed by enzymes can be monitored by examining single, broad substrate enzymes. Naphthalene dioxygenase, for example can catalyse many reactions

including mono- and di-hydroxylations and N-dealkylations against organic compounds (Resnick et al., 1996; Ellis et al., 2000). Also, individual microorganisms have been shown to possess sets of genes for the breakdown of a given pollutant such as QACs and aromatic hydrocarbons (Ferrero, 2002). Microorganisms have the metabolic capabilities to degrade more than one pollutant or use extracellular and intracellular enzymes for the metabolism of a single substrate (Kahng et al, 2001; Van Hamme et al. 2003).

The degradation of BDHAC by bacteria involving C-alkyl-N fission reaction (Figure 4.2), may have been catalyzed by an oxygen independent dehydrogenase, as has been described for benzyldimethylalkylammonium chloride (Patrauchan and Oriel 2003) alkyltrimethylammonium salts (van Ginkel 1996) and dodecyldimethylamine (Kroon et al. 1994). Presumably, other products of C-alkyl-N bond fission would be correspondent alkanals, which might be further converted via β -oxidation pathway (van Ginkel 1996).

4.6 Conclusion

This study on the identification of metabolites from BDHAC biodegradation revealed the formation of benzyldimethylamine (BDAM) via C-alkyl-N bond fission. It proved that growth of the strains of bacteria at the different concentrations of BDHAC was accompanied by biodegradation.

CHAPTER 5

DISCUSSION OF RESEARCH FINDINGS

5.1 Discussion

5.1.1 Biodegradation and Bioremediation

The process of bioremediation involves the degradation of chemical substances by bacteria and other microorganisms. It utilizes the metabolic versatility of microorganisms to degrade recalcitrant pollutants. Its goal is to transform organic pollutants into harmless end products or mineralise the pollutants into carbon dioxide and water. The technique has been shown to have potential for broad applications in terrestrial, freshwater and marine environments for treating soils and sediments contaminated with oil and other substances. (Atlas 1995; Alexander, 1999).

Biodegradation is a viable bioremediation technology for pollutants. It has long been known that microorganisms degrade environmental pollutants in various matrices and environments. The predominant degraders of organo-pollutants in contaminated sites are chemo-organotrophic species (species deriving energy from the oxidation of organic compounds) which are capable of using a large number of xenobiotics (chemicals found in much higher concentrations than usual) as sources of carbon and as electron donors for the generation of energy (Houghton and Shanley, 1994).

Surfactants such as QACs have been found in the aquatic environment mainly due to their widespread usage and discharge of effluents into surface waters. QACs discharged in produced water to the marine environment are strongly associated with particulates or sediment. They can be degraded under aerobic conditions and elevated concentrations of these compounds and their degradation products may affect organisms in the environment

(Ying, 2006). Adaptation of aquatic microbial communities to QACs (to use them as a carbon source) can be divided into fate and effect components. The fate adaptation component has received some attention (Spain, et al., 1980; Spain and Van Veld, 1983) and refers to a process whereby the rate of biodegradation of a chemical is significantly increased as a result of prior exposure. The fate adaptation processes are important since they facilitate the rapid removal of xenobiotics from the environment and minimise the potential for exposure of sensitive aquatic biota to these chemicals.

5.1.2 Bacterial Degradation of Quaternary Ammonium Compounds

Several authors have reported the isolation of bacteria capable of degrading QACs. Van Ginkel et al, (1992) isolated a *Pseudomonas* strain B1 capable of degrading hexadecyltrimethylammonium chloride from activated sludge. Also Nishiyama et al., (1995) isolated *Pseudomonas fluorescens* capable of degrading alkyltrimethylammonium salt from activated sludge. In another study Patrauchan and Oriel (2003) isolated *Aeromonas hydrophilia* sp. K capable of utilising benzyldimethylalkylammonium chloride from polluted soil.

The isolation of Gram-negative bacteria capable of utilising QACs has been reported. Evidence has been found that links resistance to QAC in Gram-negative bacteria to an increase in lipid, presumably within the cell envelope (Chaplin 1952 and MacGregor et al, 1958). In addition, Russel and Chopra (1996) reported that QACs have little effect against gram-negative bacteria due to thickened structure of their cell wall. Also, studies by Dean-Raymond and Alexander, (1977); Nishihara et al., (2000); van Ginkel (1996); and van Ginkel et al, (1992), reported that most isolated bacteria able to degrade quaternary ammonium compounds were representatives of the genus *Pseudomonas*. Dean-Raymond and Alexander (1977) reported that a mixture of *Pseudomonas* and *Xanthomonas* isolated from soil and sewage grew in a medium consisting of decyl-trimethylammonium salt as a sole carbon source, while *Xanthomonas*, alone, in the presence of yeast extract and casamino acids, oxidized the terminal carbon of the long alkyl chain of the salt. Also, Langsrud and Sundheim (1997) and Langsrud et al., (2003) observed that *Pseudomonas* sp., can adapt to sanitizers used in food-processing industries and that they can adapt to

survival in media containing high concentrations of QACs. Nagai et al., (2003) isolated *Pseudomonas aeruginosa* strain PFRB, from a stock solution of benzalkonium chloride, and exposed the strain to QACs. They observed that the strain gradually showed high-level resistance to hexadecyltrimethylammonium bromide. Also, Langsrud et al., (2003) observed that *Pseudomonas aeruginosa* ATCC 15422, and *Pseudomonas* sp. isolated from cold-stored chicken developed tolerance to high concentrations of QACs after several rounds of re-inoculations in Mueller-Hinton broth containing gradually higher amounts of QACs. The Pseudomonads are aerobic gram-negative rods e.g. *Pseudomonas putida* and *P.fluorescens*. They seem to have the highest QAC degradative potential (Haughton and Shanley, 1994). Other important aerobic Gram-negative degraders are found within the genera *Comamonas*, *Burkholderia* and *Xanthomonas*. Resistance to quaternary ammonium compounds among Gram-negative species has been observed by (Russell et al. 1986; Szumala and Pernak 1986; Jones et al.1989; Russell and Chopra 1996; Langsrud and Sundheim 1997).

The present study focused on the isolation of heterotrophic bacteria (i.e. those bacteria that require an organic carbon source for growth, deriving energy and carbon from organic compounds) from the marine environment that were capable of metabolizing BDHAC (a quaternary ammonium compound containing a benzyl ring) at concentrations higher than that used in corrosion inhibitor formulations. Bacteria were isolated from marine sediment, following enrichment in the presence of 0.05 mg/ml of BDHAC, and were characterised by catalase, oxidase, motility and micromorphological screening tests. Tentative identification of the strains was by sequencing of their 16S-rDNA genes. The isolated bacteria were of several genera other than Pseudomonads. Hence, the degradation of quaternary ammonium compounds seems not to be an exclusive trait of members of the genus *Pseudomonas* and *Xanthomonas* as suggested by Dean-Raymond and Alexander, (1977); Nishihara et al., (2000); and van Ginkel (1996).

Identification and characterization of Bacterial Isolates

The bacteria isolated from marine sediment were identified as: *Bacillus subtilis*, *Micrococcus luteus*, *Bacillus niabensis*, *Thalassospira* sp., *Rhodospirillaceae* sp.,

Staphylococcus equorum, *Sporosarcina* sp., and *Actinobacterium*. Cells of *B.niabensis*, *B. subtilis*, *Actinobacterium*, *Micrococcus luteus* and *Staphylococcus equorum* were Gram positive, while *Thalassospira* sp., *Rhodospirillaceae* sp., and *Sporosarcina* sp. were Gram-negative. The Gram stain results were in agreement with other identifications carried out. The isolated strains of bacteria were exposed to BDHAC in growth medium.

B.subtilis and *B.niabensis* are members of the genus *Bacillus*, which have the ability to form tough, protective endospores, allowing the organisms to tolerate extreme environmental conditions (Nakano and Zuber 1998). *Actinobacteria* can be terrestrial or aquatic. They include some of the most common soil life, freshwater and marine life, playing an important role in decomposition of organic materials (Servin et al., 2008). The genus *Staphylococcus* includes thirty-three species found worldwide but they are a small component of soil microbial flora (Holt 1994; Ryan and Ray 2004). Sundheim, et al., (1992) reported that regular exposure to QACs of Gram-positive bacteria, such as *Staphylococcus aureus*, brought about their adaptation and resistance to these chemicals.

Rhodospirillaceae are a family of Proteobacteria often found in anaerobic aquatic environments, such as mud and stagnant water, although they are able to survive in air. Proteobacteria are Gram-negative, with an outer membrane that is composed of lipopolysaccharides. (Madigan et al., 2004). *Sporosarcina* sp. belongs to the family Planococcaceae, which are aerobic endospore-forming bacteria found in surface water and sediment. *Thalassospira* species is from the genus *Thalassospira* and are also found in the marine environment (Lopez-Lopez et al. 2002).

In the light of literature cited, there have been no reports on the biodegradation of QACs by any of the strains of bacteria identified in the present study.

Growth of bacterial strains and BDHAC biodegradation

Following isolation and identification of strains in the present study, biodegradation experiments were carried out by incubating them in the presence of BDHAC at concentration between 0.05- 4 mg/ml (50-4000 mg/l) and controls without BDHAC. Optical density (OD₆₅₀) measurements were used to follow growth and the amount of residual BDHAC in the growth medium was quantified using Colorimetric Dye Binding Assay and

Electrospray Ionisation Mass spectrometry when the equipment was available. After the amount of BDHAC was quantified, the products formed as a result of BDHAC transformation were investigated. Growth of the strains was generally minimal at the concentrations of BDHAC tested. Minimal growth may have been as a result of prolonged lag phase and not due to toxicity of BDHAC to the bacteria. Growth of the isolated bacteria at BDHAC concentrations 0.05- 4 mg/ml observed in the present study was in agreement with reports from studies by Adair et al. (1969) and Dean - Raymond and Alexander, (1977) which stated the ability of *P. aeruginosa* to survive and grow in solutions containing 1,000 µg of commercially available benzalkonium chloride (BC) per ml (1mg/ml BC). These observations support the assumption that high concentrations of QACs are not toxic to the growth of some bacteria.

To ensure that the minimal growth of the bacteria observed, was not due to toxicity of BDHAC, marine broth was used in subsequent experiments as the main constituent of growth medium and incubation time was reduced first from 28 days to 7 days and then to 120 hours and the starting OD was set at 0.08 nm. Also further investigations were carried out to check for the effect of methanol on the strains, since methanol was used in the preparation of BDHAC solutions. Growth results obtained for the strains in marine broth were higher than those observed for minimal salt medium and 2% of methanol used as solvent for BDHAC showed no effect on growth of the strains. High growth levels were observed for high concentrations of BDHAC as compared to the controls that lacked BDHAC, suggesting that the Quat was a source of carbon and energy for the bacteria. BDHAC may not have been the sole source of carbon and energy for the growth of the bacteria, but comparing growth of cultures with high concentrations of BDHAC with controls that lacked BDHAC, it was observed that more BDHAC disappeared in the course of time. Recoveries of residual BDHAC were also carried out to ensure that losses observed were due to biodegradation.

As demonstrated by ESI-MS/MS and CDBA analysis, the extents of BDHAC degradation reached up to 90% during the periods of incubation. BDHAC contains a benzyl group and some authors have reported the degradation of a benzyl ring containing QAC although the organisms that utilised the QACs were not isolated. Gawel and Huddleston, (1972) recorded that 95% of 10 mg/l tetradecylbenzyltrimethylbenzyl ammonium chloride

disappeared in 2 days in a shake flask culture, and confirmed the disappearance of the QAC based on colorimetric and UV spectrophotometric analysis. Arpino and Ruffo, (1976) observed that 100% of 20 mg/l alkyldimethylbenzylammonium type Quat was degraded in 11 days while Pitter et al., (1976) noted that 96% of 500 µg/ml of an alkyldimethylbenzylammonium type Quat was utilised in 20 days. Results from a study by Ruiz Cruz, (1979) revealed that tetradecylbenzyltrimethylammonium was degraded in 3-4 days and Ghisalba and Kuenzi, (1983) observed the biodegradation of alkyltrimethylammonium type quaternary ammonium surfactant by 100% in 215 hours of incubation. The authors did not state the concentrations of the quaternary ammonium compounds. All these studies indicate that quaternary ammonium based surfactants were biodegradable at least at a primary level. Primary biodegradation is the disappearance of a compound to an extent that either specific characteristic properties are no longer evident, or no longer responds to a compound specific analytical procedure.

Patrauchan and Oriel, (2003) also reported that Benzylalkyldimethylammonium chloride added to final concentrations of 30–400 mg/l in an enrichment medium and *A. hydrophila* sp. K isolated from polluted soil was able to degrade the recalcitrant xenobiotic. *Aeromonas hydrophila* is a heterotrophic, gram-negative, rod shaped bacterium, mainly found in areas with a warm climate. This bacterium can also be found in fresh, salt, marine, estuarine, chlorinated, and un-chlorinated water. *A. hydrophila* can survive in aerobic environments. *A. hydrophila* was similar to *Thalassospira* sp., *Rhodospirillaceae* sp., and *Sporosarcina* sp. investigated in the present because it was a Gram-negative rod.

These observations support the fact that benzyl ring containing QACs such as BDHAC can be degraded by bacteria. Many other degradation studies reported in the literature have utilised QACs without a benzyl ring. For example, Pitter and Svitalkova (1961) reported the biodegradation of 100% of an alkyltrimethylammonium type quaternary ammonium surfactant in 8 hours of incubation and Van Ginkel et al., (1992) reported that *Pseudomonas* sp. strain B1, isolated from activated sludge, grew well on hexadecyltrimethylammonium chloride using the compound as a carbon and energy source.

However, QACs have been observed to have bacteriocidal properties and Russel et al. (1992a) reported the use of QACs as anti-microbial agents. These surfactants have been observed to disrupt the structure of bacterial cell membranes (Takasaki et al. 1994a, b)

and have been used for cattle-shed disinfection and other anti-microbial applications (Cursons et al. 1980; Preston 1983; Furuta et al. 1991; Seki et al. 1993; Chen et al. 1995). Some studies have also reported that degradation of QACs by bacteria can only happen when they are present at low concentrations (Anon 1993). However, results obtained from this study utilising up to 4 mg/ml BDHAC, suggested that *Bacillus subtilis*, *Bacillus niabensis*, *Thalassospira sp.* and *Sporosarcina sp.* isolated from marine sediment were less susceptible to high concentrations of the QAC when they were continuously exposed to these concentrations in the growth medium. Continuous exposures of pure cultures of heterotrophic bacteria to high concentrations of QACs have been shown to make the strains less susceptible (Ventullo and Larson 1986; Vesteege and Shorter, 1992 and McBain et al. 2004) and adaptation to QACs can be enhanced by continuous exposure. It has also been observed that regular exposure of *Staphylococcus aureus* and *Pseudomonas spp.* to QACs brought about adaptation and resistance to these compounds (Sunheim, et al. 1992; Langsgrud and Sunheim 1997; Langsrud et al, 2003). The authors suggested that *Pseudomonas spp.* can adapt to survival in the presence of high concentrations of QACs.

In the light of the results obtained in this study for BDHAC biodegradation, an assumption was made that bacterial strains isolated from marine sediments were able to degrade BDHAC because of the possible presence of substrates in the growth medium which may have formed a complex with the QAC thereby limiting its concentration in the aqueous phase. Growth on toxic compounds such as high concentration of QACs in batch cultures is possible in the presence of silica gel (van Ginkel, 1992). The author reported that silica gel acted as an adsorbent which slowly released hexadecyltrimethylammonium chloride during the growth of bacteria, thus preventing inhibitory concentrations.

5.1.3 Identification of Products from BDHAC Biodegradation

Biodegradation is environmentally acceptable when a chemical is partially degraded, provided that products formed are considered to have no harmful effects on the environment. This requires that the by-products of the bacterial degradation be known. The mechanism of biodegradation of chemicals in the marine environment has become one of the fundamental issues in understanding environmental safety of chemicals. An

investigation was therefore carried out to determine possible products of degradation of BDHAC by the isolated strains of bacteria. Analysis of the samples obtained from the degradation experiments by ESI-MS/MS showed benzyldimethylamine as a product of BDHAC transformation, which suggested that the initial attack on BDHAC molecules was a central fission of a C-alkyl-N bond. This finding supports the view that C-alkyl-N fission of the alkyl compounds by micro-organisms is a general strategy of gaining access to the alkyl chains (van Ginkel, 1995; Patrauchan and Oriel, 2003).

Several studies on the biodegradation pathways of QACs have been carried out and results of these studies have been similar. For example, Hampton and Zatman (1973) investigated the utilisation of tetramethylammonium chloride by *Pseudomonas* sp. The authors observed that the metabolism of this compound was initiated by the splitting of the C-alkyl-N bond, producing methanal and trimethylamine. In another study on the biodegradation of hexadecyltrimethyl ammonium chloride, van Ginkel et al., (1992) suggested a central fission of the C-alkyl-N bond as the first step of the biodegradation process with production of hexadecanal and hexadecanoate as metabolites, and these metabolites were all utilised by *Pseudomonas* sp. The mechanism of degradation of alkylbenzyldimethyl ammonium chloride was also proposed by Patrauchan and Oriel, (2003). They reported the complete metabolism of dodecyl- and tetradecyl-benzyldimethyl ammonium chloride (BAC) by *Aeromonas hydrophilia* sp. K. In their study they suggested that the central C-alkyl-N bond fission was first carried out by an oxygen dependent dehydrogenase as suggested for alkyltrimethyl ammonium compounds by van Ginkel et al., (1992). The intermediates formed from the biodegradation process were benzylamines and benzoic acid. Results from their investigation revealed that C-benzyl-N bond fission was not feasible. The observation made by Patrauchan and Oriel, (2003) applies to the present study as both BDHAC and BAC contain a C-benzyl-N bond. Takenaka et al., (2007) reported that degradation of dodecyltrimethyl ammonium chloride by *Pseudomonas* sp. strain 7-6 occurred via dual pathways; an initial attack on the C-alkyl-N bond, and also degradation via cleavage of a C-methyl-N bond, producing dodecyldimethyl amine as an intermediate.

Generally, observations from previous studies on the degradation pathways of cationic surfactants such as QACs including those with a C-benzyl-N bond or a C-methyl-

N bond, suggest that biodegradation commences with cleavage of the C-alkyl-N and that further degradation of alkanals formed will be via β -oxidation for complete mineralization to occur (van Ginkel and Kolvenbach, 1991).

It is important to state here that the degradation of BDHAC by bacteria isolated from marine sediments occurred via C-alkyl-N bond cleavage because the surfactants formed micelles at the concentrations tested. Micelles were formed because the concentrations of the surfactant were higher than its critical micelle concentration (CMC). The Critical Micelle Concentration (CMC) is defined as the concentration of surfactants above which micelles are formed. Marcotte et al., (2005) in a study on permeability and thermodynamics of quaternary ammonium surfactants-phosphocholine vesicle system reported the CMC of BDHAC as 0.004 Mm.

When BDHAC was introduced into the growth medium during the degradation experiment at different concentrations above the CMC, it initially partitioned into the interface, reducing the free energy of the medium by lowering the energy of the interface and removing its hydrophobic parts (the part with the benzyl ring) from contacts with bacteria and the growth medium. Subsequently, when the surface coverage of BDHAC increased and the surface free energy decreased, it aggregated into micelles. Once the CMC of a surfactant for example BDHAC is reached, any further addition of the surfactant will only increase the number of micelles, Fuchs-Godec, R. (2006). Surfactants such as BDHAC have the ability to form micelles in solution. This is due to the presence of both hydrophobic (side chain with the benzyl ring) and hydrophilic (alkyl side chain) groups in their molecule. The formation of micelles in solution gives BDHAC its detergency and solubilisation properties (Haigh, 1996). When micelles are formed a proportion of the surfactant molecule remains outside of the micelle structure, and there is an equilibrium exchange of the surfactant molecule between the micelle and the solvent. For BDHAC breakdown to occur at the C-N-alkyl bond site there is the possibility that this will take place when the surfactant molecules are removed from the micelle, or else this bond site would be protected by the micelle.

In summary, the present study showed that the strains of heterotrophic bacteria isolated from sediments from the Firth of Forth at Cramond beach, Scotland were able to

individually degrade the alkyl side chain of BDHAC when it was present at concentrations ranging from 0.05- 4 mg/ml greater than its CMC. The aromatic ring was removed from the growth medium by the formation of micelle.

High BDHAC concentrations may impact on the marine environment as a result of discharges of waste at different stages of the oil production process. The continuous discharge of produced water along with production chemicals during the routine operation of production platforms is a constant source of toxicity to the marine environment and the biodegradation of recalcitrant compounds in produced water that find their way into the environment is an important consideration with regard to the fate of these compounds in the environment and the self-cleaning attribute of the natural environment (van Ginkel et al., 1992)

5.1.4 Application of the Research Findings in Bioremediation

Biodegradation as a method of bioremediation is very useful in oil exploration and production processes in the oil industry due to its low operational cost and the reduced environmental impact when compared to other conventional physical and physicochemical remediation methods (Xu et al. 2003)

In offshore oil exploration and production, corrosion is one of the most serious problems as production facilities are subject to corrosive attack by carbon dioxide, hydrogen sulfide and other aggressive constituents that may be present in reservoir fluids. To mitigate these problems, production chemicals (corrosion inhibitors) containing QACs such as BDHAC are added to topside equipment at various stages in the production process. These residual chemicals and other residues are often discharged in produced water to surface waters/sediment and may cause widespread contamination of the marine environment (Hudgins 1991).

The marine environment is extremely sensitive and is a habitat for different species, forming different biomes important to environmental quality (Cubitto et al, 2004). Therefore marine environmental protection and sediment remediation processes are areas receiving increasing attention. The present research was stimulated by the fact that petroleum production takes place at offshore installations in the North Sea, and the marine

environment in the vicinity of these platforms has been polluted by produced water discharges from these installations. The purpose of this work was to contribute to a better understanding of the ability of microorganisms to degrade benzalkonium quaternary ammonium salts present in these discharges. Tolerance of bacterial cultures to high concentrations of BDHAC observed in this research is potentially useful as it will contribute to an understanding of the potential of the isolated strains of bacteria in bioremediating BDHAC contaminated sites.

Bioremediation is a process by which bacteria and other microorganisms degrade chemical substances. It can be used in decontaminating polluted sediment by the addition of exogenous microbial populations, or the stimulation of indigenous populations or manipulation of contaminated media using techniques such as aeration or temperature control (Hoff, 1993; Atlas, 1995; Swannell et al., 1996). The use of microorganisms has been successfully applied in the bioremediation of contaminated sites (Atlas, 1995). Several studies have investigated the use of bioremediation for cleanup in seawater, freshwater and terrestrial areas. The technique has potential for broad applications in terrestrial, freshwater and marine environments for treating soils and sediments contaminated with chemicals (Hoff, 1993). It is important to address the pollution of the marine environment as soon as possible as contamination can damage fishery resources and affect the health of those animals and humans that consume contaminated fish (Krahn & Stein, 1998).

Several bioremediation techniques are used to accelerate the rates of biodegradation of cationic surfactants in the environment (Irwin, 1996). Populations of bacteria can be stimulated through the addition of nutrients or other materials and exogenous bacterial populations can be introduced to a contaminated environment. The addition of exogenous bacteria to a contaminated site is known as bio-augmentation. For the process of bio-augmentation, genetically altered bacteria can be used and contaminated sites such as marine sediment can be seeded with these microbial cultures (Irwin, 1996). 'Most microorganisms considered for seeding are obtained by enrichment cultures from previously contaminated sites' (Atlas, 1995). Marine sediment contaminated with BDHAC can be seeded with the four strains of bacteria investigated in this report to initiate bioremediation of the sediment. However, it is not well established that these strains of bacteria will degrade high BDHAC concentrations in the presence of other less toxic

substrates in the marine environment. Bioremediation is an alternative to the physical removal and destruction of pollutants. Although this technique requires characterization of the nature of site, contaminants present, microorganisms and their activities, it is cost effective and therefore studies on the large scale cultivation of contaminant-specific bacteria reported in the BDHAC biodegradation studies should be considered. These large scale bacterial cultures when seeded to contaminated sediments have the potential of mineralising BDHAC to harmless products.

Finally, further studies will enable operators and regulators in the oil and gas industry to understand the fate of BDHAC in the environment when the compound is used in corrosion inhibitor formulations. This understanding will assist environmental risk assessment of oil production facilities and, following platform decommissioning, may provide a basis for a sediment remediation strategy.

5.2 Conclusion

Based on all of the observations made in the present study, the following conclusions can be made:

- (1) Gram-negative and Gram-positive bacteria isolated from marine sediment could degrade BDHAC when it was present at concentrations higher than that dosed in corrosion inhibitor formulations.
- (2) The strains of bacteria capable of degrading BDHAC were of several genera but not *Pseudomonas*. The study therefore revealed that QAC degradation was not an exclusive trait of members of the genus *Pseudomonas*.
- (3) The strains of bacteria could grow in medium containing BDHAC at concentrations of 1, 2 and 4 mg/ml and bacteriocidal properties of the QAC was not evident at these concentrations. This was because the concentrations tested were above the CMC of BDHAC and the micelles formed protected the surfactant molecule from the bacteria.

(4) In minimal medium and marine broth containing BDHAC, bacterial growth was accompanied by degradation. Benzyldimethylamine was observed as a product of the BDHAC transformation. This suggested that the initial attack on BDHAC molecules was a central fission of a C-alkyl-N bond. Results also suggested that C-benzyl-N bond fission did not occur as the first step of the biodegradation process due to micellisation of BDHAC at concentrations above its CMC.

5.3 Future Work

Although results obtained from this research suggest that some bacteria present in marine sediment are capable of degrading a quaternary ammonium salt (BDHAC) which is a proprietary corrosion inhibitor ingredient, the biodegradation process may not have gone beyond the primary level i.e. the yield of benzyldimethylamine as a product of the surfactant transformation and the inability to find other metabolites such as N-benzylmethylamine (BMAM), benzoic Acid (BA), and benzaldehyde (BAH). This may have been because of BDHAC's amphiphilic (i.e., both hydrophilic and hydrophobic) property and the limited enzymatic ability of individual bacteria that were present in the growth medium. Only a few known surfactants, alkyl sulphonates, alkyl sulphates, and alkyl amines have been reported to be ultimately degraded by a single microorganism (van Ginkel, 1996).

For this reasons, future work should be carried out using consortia of microorganisms. Some studies have shown that consortia are highly efficient, as well as necessary, for the complete degradation of QACs (van Ginkel, 1996). Such consortia of microorganisms are classified as either commensal (one benefits, whereas the other is not affected) or synergistic (both organisms take part in the degradation process). A good example of commensalism can be seen in the degradation of hexadecyltrimethyl ammonium chloride reported by van Ginkel et al., (1992). A *Pseudomonas* sp. was observed to have attacked the C-alkyl-N bond of the QAC to produce trimethylamine which supported the growth of methylotrophs. Also, Patrauchan and Oriel, (2003) observed that *Aeromonas hydrophilia* sp. K was not capable of utilizing alkyl dimethylamine produced from alkyl benzyldimethyl chloride transformation. The complete degradation of

the QAC required three bacteria that metabolized the alkyl chain, the aromatic moiety, and dimethylamine (van Ginkel and Kolvenbach, 1991). In addition the degradation of decyltrimethyl ammonium bromide investigated by Dean-Raymond and Alexander (1977) suggested a synergistic relationship between the bacterial strains in the growth medium. Decyltrimethyl ammonium chloride was extensively mineralised by two bacteria *Pseudomonas sp.* and *Xanthomonas sp.* together, but the strains did not grow to a good level when they were cultured in isolation. These observations suggest that products from partial metabolism of QACs by one strain can be necessary for the survival and growth of another.

There were several limitations to the present research. The ESI-MS analytical technique could not identify other metabolites apart from N, N-dimethylbenzylamine. There was also constant breakdown of the mass spectrometer during experiments. Future work should aim to identify other products of BDHAC transformation based on other analytical techniques such as Gas Chromatography/Mass Spectrometry (GC-MS), High Performance Liquid Chromatography (HPLC), Ion-Chromatography and ^1H -Nuclear Magnetic Resonance (^1H -NMR) Spectroscopy. Dean-Raymond and Alexander (1977) used a Finnigan Gas Chromatograph-Mass Spectrometer equipped with data processing system to identify the metabolites of 10 Quaternary ammonium compounds. Also, Takenaka et.al, (2007), identified the metabolites of n-dodecyltrimethylammonium chloride (DTAC) by Gas Chromatography-Mass Spectrometric analysis, Ion-Chromatography and High-Performance Liquid Chromatography (HPLC) with an electrical conductivity detector and an ion exchange column. ^1H -Nuclear Magnetic Resonance (^1H -NMR) Spectroscopy was also used by Nishihara et al., (2000) for the quantification and identification of didecyltrimethylammonium chloride (DDAC) and its degradation products and Patrauchan and Oriel (2003) analysed the products of Benzylalkyldimethylammonium chloride (BAC) transformation by reverse phase HPLC. According to these authors, these analytical techniques were suitable for the complete identification of the products of QAC metabolism.

Besides the shake flask method, another biodegradation method such as the closed bottle BOD method should be adopted in the investigation of BDHAC biodegradation. The closed bottle test (CBT) is recommended as a first, simple test for the assessment of the

ready biodegradability of organic compounds (OECD 301D, 1992). These method that measure oxygen uptake was used by Dean-Raymond and Alexander (1977) to test for the biodegradation of 10 quaternary ammonium compounds and the results of the BOD tests confirmed that quaternary ammonium compounds are subject to biodegradation.

Also, effort should be made at isolating the strains of bacteria under investigation anaerobically and at the same time check if the isolated bacteria can utilise BDHAC in an anaerobic atmosphere. Ulas, (2009) in a study on the fate and effect of quaternary ammonium compounds in biological systems, demonstrated the degradation of benzalkonium chloride (BAC) in bioassays performed using a mixed enrichment culture under fermentative and nitrate reducing conditions. In the presence of fumarate, BAC was completely transformed to a compound which was presumed to be succinyl-benzalkonium (SBA) based on LC/MS analysis. SBA was not further degraded during the fumarate fermentation and in the absence of nitrate reduction. This is the only report available on QAC transformation under anoxic/anaerobic conditions.

Finally, it would also be interesting to carry out detailed assays to examine the enzymes that participate in the biodegradation of BDHAC to propose a pathway for BDHAC transformation. An enzyme that has been reported to be involved in the initial step of *n*-dodecyltrimethylammonium chloride (DTAC) metabolism by *Pseudomonas sp.* strain 7-6 was a monooxygenase (Takenaka et al., 2007). Also Nishihara et al. (2000) reported the activity of a monooxygenase as the first enzyme in the biodegradation of didecyltrimethylammonium chloride (DDAC) by *Pseudomonas fluorescens*. In addition, van Ginkel (1992), showed evidence in a study of the metabolism of hexadecyltrimethylammonium chloride *Pseudomonas sp.* strains B1. He stated that the oxidation of the alkyl chain of the QAC was first catalysed by an oxygenase. However, Patrauchan and Oriel, (2003) suggested that the C-alkyl-N bond fission observed in the biodegradation of dodecyl- and tetradecyl-bezyldimethyl ammonium chloride by *Aeromonas hydrophila sp. K* was first carried out by an oxygen dependent dehydrogenase. Enzyme assays are therefore needed to determine the specific enzyme(s) that catalyse the fission of the C-alkyl-N bond of BDHAC to produce benzyldimethylamine.

REFERENCES

- Adair, F., Geftic, G., and Gelzer, J. (1969) Resistance of *Pseudomonas* to quaternary ammonium compounds. I. Growth in benzalkonium chloride solution. *Appl. Microbiol.* 18:299-302.
- Ahlstroem, B., Chelminska-Bertilsson, M., Thompson, A. and Edebo, L. (1997) Submicellar complexes may initiate the fungicidal effects of cationic amphiphilic compounds on *Candida albicans*. *Antimicrob. Agents Chemother.* 41:544–550.
- Ahlström, B., Thompson, R. and Edebo, L. (1999). The effect of hydrocarbon chain length, pH and temperature on the binding and bactericidal effect of amphiphilic betaine esters on *Salmonella typhimurium*. *APMIS* 107:318–324
- Ahmed, I., Arima, J., Ichiyanagi, T., Sakuno, E. and Mori, N (2009) Isolation and characterization of 3-*N*-trimethylamino-1-propanol-degrading *Rhodococcus* sp. strain A2. *FEMS Microbiology Letters* 296 (2), pp 219–225.
- Ahmed, I., Arima, J., Ichiyanagi, T., Sakuno, E. and Mori, N (2009) Isolation and Characterization of 3-*N*-Trimethylamino-1-Propanol Degrading *Arthrobacter* sp. Strain E5 *Research Journal of Microbiology* 4(2) pp. 49-58.
- Ahmed, I., Arima, J., Ichiyanagi, T., Sakuno, E. and Mori, N (2010) Isolation and characterization of homocholine-degrading *Pseudomonas* sp. strains A9 and B9b *World J Microbiol Biotechnol* (2010) 26:1455–1464
- Alexander, M., (1973) Nonbiodegradable and other recalcitrant molecules. *Biotechnol Bioeng* 15:611-647
- Alexander M. (1999) *Biodegradation and Bioremediation*, 2nd edition Academic Press, New York.

REFERENCES

- Ali, S., Henry, L., Darlington, J. and Occapinti, J. (1999) "Novel Filtration Process Removes Dissolved Organics from Produced Water and Meets Federal Oil and Grease Guidelines, 9th Produced Water Seminar, Houston, TX.
- Annweiler, E., Richnow, H., Antranikian, G., Hebenbrock, S., Garms, C., Franke, S., Franke, W. and Michaelis, W. (2000). Naphthalene degradation and incorporation of naphthalene derived carbon into biomass in the thermophile *Bacillus thermoleovorans*. *App Environ Microbiol* 66: 518-523
- Anon. (1992) In Biodegradation and Bioaccumulation Data of Existing Chemicals Based on the CSCL Japan. Chemical Inspection Testing Institute Japan. pp. 3138-3139. Tokyo: Japan Chemical Industry Ecology-Toxicology and Information Center.
- Anon (1993) In Environmental Fate Data Base 'BIODEG'. Syracuse, NY: Syracuse Research Corporation.
- Arpino, A. and Ruffo, C., (1976) Contribution to the microdetermination of cationic surfactants. *Riv. Ital. Sostanze. Grasse* 53:395-399
- Atlas, M. (1995). Petroleum Biodegradation and Oil Spill Bioremediation. *Marine Pollution Bulletin* 31, 178-182.
- Bansal, K., and Caudle, D. (1999) "Interferences with Processing Production Water for Disposal," 9th Produced Water Seminar, Houston, TX.
- Bruins, A. (1998). Mechanistic aspects of electrospray ionization. *J Chromatogr A*; 794:345-57.
- Beveridge, C., Parr, A., Smith, M., Kerr, A., Cowling, M. and Hodgkiess, T. (1998) The effect of benzalkonium chloride concentration on nine species of marine diatom. *Environmental Pollution* 103, 31-36.

REFERENCES

- Boething, R. (1984) Environmental fate and toxicity in wastewater treatment of quaternary ammonium surfactants. *Water Research* 18, 1061–1076.
- Bore, E., He´braud, M., Chafsey, I., Chambon, C., Skjæret, C., Moen, B., Møretrø, T., Langsrud, Ø., Rudi, K., and Langsrud, S. (2007) Adapted tolerance to benzalkonium chloride in *Escherichia coli* K-12 studied by transcriptome and proteome analyses *Microbiology*, 153, 935–946
- Brendehaug, J., Johnsen, S., Byrne, K., Gjose, A., Eide, T., and Aamot, E. (1992) Toxicity Testing and Chemical Characterisation of Produced Water- A preliminary Study. *In* Produced Water. Edited by Ray, J. and Engelhart, F. Plenum Press, New York, pp 245-256.
- Bruins, A. (1998) Mechanistic Aspects of Electrospray Ionization. *J Chromatogr A*; 794:345–57.
- Brunt, J., Austin, B., (2005) Use of a probiotic to control lactococcosis and streptococcosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish Dis.* 28, 693-701.
- Callely, A., and Forster, C. (1977) Stafford, Treatment of industrial effluents, Hodder & Stoughton, London.
- Cain, R. (1976). Surfactants biodegradation in wastewaters, p. 283-327. *In* A. G. Callely, C. F. Forster, and D. A. Stafford (ed.), Treatment of industrial effluents. John Wiley & Sons, Inc., New York.
- Challaghan, D. (1991) Chemicals in Oil Production: Producers Perceived Needs. *In* Chemicals in the Oil Industry. Edited by Ogden, P. Royal Society of Chemistry, pp 96-106.
- Chaplin, C. (1952) Bacterial resistance to quaternary ammonium disinfectants. *J. Bacteriol.* 63:453-458.

REFERENCES

- Chen, T., Dwyre-Gygax, R., Smith, S. and Breuil, C. (1995) Enzyme-linked immunosorbent assay for didecyldimethylammonium chloride, a fungicide use by the forest products industry. *Journal of Agricultural and Food Chemistry* 43, 1400-1406.
- Chipasa, K., and Medrzycka, K. (2004a). "Adaptive response of microbial communities to soluble microbial products." *J. Ind. Microbiol. Biotechnol.*, 31, 384–390.
- Chipasa, K., and Medrzycka, K. (2004b). "Behavior of microbial communities developed in the presence/reduced level of soluble microbial products." *J. Ind. Microbiol. Biotechnol.*, 31, 457–461
- Cline, J. (1998) "Treatment and Discharge of Produced Water for Deep Offshore Disposal," presented at the API Produced Water Management Technical Forum and Exhibition, Lafayette, LA, Nov. 17-18.
- Collins, C. and Lyne, P. (1984) *Microbiological Methods*. 5th Ed. Butterworth and Co. Publisher Ltd. London 331 - 345.
- Cruickshank R, Duguid JP, Marmion BP, Swain RHA (1975). *Medical Microbiology*, Vol. 2. The Practice of Medical Microbiology 12th Edn. Churchill Livingstone, Edinburgh. pp. 273-283.
- Cubitto, M., Morán, A., Commendatore, M. Chiarello, M., Baldini, M., Siñeriz, F. (2004) Effects of *Bacillus subtilis* O9 biosurfactant on the bioremediation of crude oil-polluted soils. *Biodegradation* 15:281–287
- Cursons, R., Brown, T. and Keys, E. (1980) Effect of disinfectants on pathogenic free-living amoebae: axenic conditions. *Applied and Environmental Microbiology* 40, 62-66.

REFERENCES

- Danish EPA, (2003) "PAHs in the Marine Environment, Ministry of Environment and Energy," Danish Environmental Protection Agency, Faktuel.
- Dean-Raymond, D., and Alexander M. (1977) Bacterial metabolism of quaternary ammonium compounds, *Appl. Environ. Microbiol.* 33 1037–1041.
- Desbordes, J. and Jourdan, R. (1974) Influence of bacterial enzymes on molecular degradation of antiseptics utilized as preservatives in medical preparations. *Dev. Biol. Stand.* 24:77-90.
- Diamanti-Kandarakis, E., Bourguignon, J., Giudice, L., Hauser, R., Prins, G., Soto, A., Zoeller, R., Gore, A., (2009). "Endocrine-disrupting chemicals: an Endocrine Society scientific statement". *Endocr. Rev.* 30 (4): 293–342.
- Diaz, E. (2008). *Microbial Biodegradation: Genomics and Molecular Biology* (1st ed.). Caister Academic Press.
- Diaz, M. (2000). Extremely-halotolerant bacterial consortia for the biodegradation of hydrocarbons in contaminated saline environments using free immobilized cell system. A PhD thesis submitted to the Heriot Watt University, Edinburgh.
- Drancourt, M., and D. Raoult (1999) Characterization of mutations in the *rpoB* gene in naturally rifampin-resistant *Rickettsia* species. *Antimicrob. Agents Chemother.* 43:2400–2403.
- Ellis, L., Hershberger, D. and Wackett, L. (2000) The University of Minnesota Biocatalysis/Biodegradation Database: microorganisms, genomics and prediction. *Nucleic Acids Res* 28:377-379.
- E & P Forum (1994). North Sea Produced Water: Fate and Effects in the Marine Environment .Report No 2.62/204, E&P Forum, 25-28 Old Burlington St, London.

REFERENCES

- Farrington, J. and Quinn J. (1973) Petroleum hydrocarbons in Narragansett, survey of hydrocarbons in sediments and dams. *Estuarine and Coastal Marine Science*, **1**: 71-79.
- Fenger, B., Mandrup, M., Rohde, G. and Sorensen, J. (1973) Degradation of a cationic surfactant in activated sludge pilot plants, *Water Res.* ,7, 1195-1208.
- Ferrer, I., and Furlong, E., (2001). Identification of alkyldimethylbenzyl-ammonium surfactants in water samples by solid-phase extraction followed by ion trap LC/MS and LC/MS/MS. *Environmental Science & Technology* 35, 2583-2588.
- Ferrero, M., Llobet-Brossa, E., Lalucat, J., Garcia-Valdes, E., Rossello-Mora, R. and Bosch, R. (2002). Coexistence of two distinct copies of naphthalene degradation genes in *pseudomonas* strains isolated from the western Mediterranean region. *Appl Environ Microbiol* 68:957-962
- Flynn, S., Butler, E. and Vance, I. (1996). Produced water composition, toxicity, and fate. A review of recent BP North Sea Studies. In *Produced Water 2: Environmental Issues and Mitigation Technologies* (Reed, M and Johnsen, S. eds) Plenum Press, NY 69-100
- Foster, C. (1977): Bio-oxidation. In: *Treatment of Industrial effluents*. Eds: Eallely, A., Foster, C. and Stafford, D. Hodder and Soughton. London.
- Fredrickson, J., Zachara, J. and Balkwill, D. (2004). Geomicrobiology of high-level nuclear waste-contaminated vadose sediments at the Hanford site, Washington state. *Applied and Environmental Microbiology* 70 (7): 4230–4241.
- Fröhlich, J. and König, H. (1999) Rapid isolation of single microbial cells from mixed natural and laboratory populations with the aid of a micromanipulator system. *Appl. Microbiol.* 22, 249-257.
- Frost T., Johnsen, S. and Utvik, T. (1998) “Environmental Effects of Produced Water Discharges to the Marine Environment,” OLF, Norway.

- Fuchs-Godec, R. (2006). "The adsorption, CMC determination and corrosion inhibition of some N-alkyl quaternary ammonium salts on carbon steel surface in 2 M H₂SO₄." *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 280 (1-3): 130-139.
- Furuholt, E. (1996) Environmental Effects of Discharge and Reinjection of Produced Water. *In* Produced Water 2: Environmental Issues and Mitigation Technologies .Edited by Reed, M and Johnsen, S. Plenum Press, New York, pp 275-288.
- Furuta, K., Uchida, K., Fujihara, H., Shimonokado, H., Moriyama, S. and Harada. Y. (1991) The evaluation of bactericidal efficacy of a disinfectant. *Japanese Poultry Science* 28, 335-339.
- Games, L., King, J. and Larson, R. (1982) Fate and distribution of a quaternary ammonium surfactant, octadecyltrimethyl-ammonium chloride (OTAC), in wastewater treatment. *Environmental Science and Technology* 16, 483-488.
- Garcia, M., Campos, E., Munoz, F., Dalmau, M. and Sanchez-Leal, J. (1997) Adsorption of cationic surfactants on biosolids. *Jor. Com. Esp. Deterg.* 27, 343-351.
- García, M., Campos, E., Sánchez-Leal J. and Ribosa, I. (2000) Anaerobic degradation and toxicity of commercial cationic surfactants in anaerobic screening tests. *Chemosphere* 41 705–710.
- García, M., Ribosa, I., Guindulain, T., Sánchez-Leal, J. and Vives-Rego J (2001) Fate and effect of monoalkyl quaternary ammonium surfactants in the aquatic environment *Environmental Pollution*. 111,169-175.
- Gawel, L and Huddleston, R. (1972). The biodegradability of low concentrations of quaternary ammonium antimicrobials by bacteria. Presented at the American Oil Chemists' Society National Meeting, Los Angeles, April 22-23.

REFERENCES

- Gerhardt, P., Murray, R., Costilow, N., Nester, W., Wood, W., Krieg, N. and Phillips, G. (1981). *Manual of Methods for General Bacteriology*. Washington, DC: American Society for Microbiology.
- Gerike, P., Fischer, W. and Jasiak, W. (1978). Surfactant quaternary ammonium salts in aerobic sewage digestion. *Water Res.* 12:1117-1122. In laboratory and environmental systems, *Dev. Ind. Microbiol.* 24: 425-434.
- Ghisalba, O. and Kuenzi, M. (1983). Biodegradation and Utilization of quaternary alkylammonium compounds by specialized methylotrophs. *Experientia*.39:1264-1271.
- Gilbert, P., Pemberton, D. and Wilkinson, D (1990) Barrier properties of the Gram-negative cell envelope towards high molecular weight polyhexamethylene biguanides. *Journal of Applied Bacteriology* 69, 585–592.
- Giolando, S., Rapaport, R., Larson, R., Federle, T., Stalmans, M. and Masscheleyn, P. (1995) Environmental fate and effects of DEEDMAC: a new rapidly biodegradable cationic surfactant for use in fabric softeners. *Chemosphere*; 30(6):1067– 83.
- Glickman, A. (1998) “Produced Water Toxicity: Steps You Can Take to Ensure Permit Compliance,” presented at the API Produced Water Management Technical Forum and Exhibition, Lafayette, LA, Nov. 17-18.
- Grigson, S., Wilkinson, A., Johnson, P., Moffat, C. and McIntosh A. (2000) Measurement of oilfield chemical residues in produced water discharges and marine sediments. *Rapd Comm. In Mass Spec.* 14 2210-2219.
- Hadson, P. (1988) The effect of metabolism on uptake, disposition and toxicity in fish. *Aquat. Toxicol.*, 11 3-18.
- Haigh, D (1996). A review of the interaction of surfactants with organic contaminants in soil. *Sci Total Environ*; 185:161–70.

REFERENCES

- Hanson, D (1997). "EPA Releases Toxics Inventory", Chemical and Engineering News, 75(23):22-23.
- Hamilton, W. (1968). The mechanism of the bacteriostatic action of tetrachlorosalicylanilide: a membrane-active antibacterial compound. J. Gen. Microbiol. 50:441–458.
- Hampton, D. and Zatman, L.J. (1973) The metabolism of tetramethylammonium chloride by bacterium 5H2. Biochem. Soc. Trans. 1:667-668.
- Hart, B. (1982) A water quality criteria for heavy metals. Australian Governmental Heidelberg. Publishing Services, Canberra.
- Heinzel, J. (1988). The phenomena of resistance to disinfectants and preservatives. In: Payne, K (Ed.), Industrial biocides, vol. 22. Critical Report on Applied Chemistry. Wiley, Chichester, p. 52- 67.
- Henderson, S. (1999) An Investigation into the Effect on Total Toxicity Predictions of Interactions between Components of a Typical North Sea Produced Water. A PhD Thesis submitted to the Heriot Watt University, Edinburgh.
- Henriquez, L. (1992) The Use/Discharge of Oilfield Chemicals and their Ecotoxicological Aspects. A paper presented at the International Oilfield symposium, Geilo, Norway.
- Ho, C., Lam C., Chan, M., Cheung, R., Law, L., Lit, L., Ng, K., Suen, M. and Tai, H. (2003) Electrospray Ionisation Mass Spectrometry: Principles and Clinical Applications Clin Biochem Rev. 24(1): 3–12.
- Hoff, Z. (1993). Bioremediation: an overview of its development and use for oil spill cleanup. *Marine Pollution Bulletin* 29, 476-481
- Holt, J. (1994) Bergey's Manual of Determinative Bacteriology (9th ed.). Williams & Wilkins.

REFERENCES

- Houghton, J. and Shanley, M. (1994) Catabolic potential of Pseudomonads: a regulatory perspective, *Biological Degradation and Bioremediation of Toxic Chemicals*, Dioscorides Press, Portland (1994), pp. 11–32.
- Hudgins, C. (1994) Chemicals Use in North Sea Oil and Gas E&P, *Journal of Petroleum Technology*, pp 67-75
- Hudgins, C., (1991). Chemical usage in North Sea Oil and Gas Production and Exploration Operations. Report for the Norwegian Oil Industry Association. Petrotech Consultants Inc. Houston, Texas.
- Hugo, W. (1967). The mode of action of antibacterial agents. *J. Appl. Bacteriol.* 30:17-50.
- Husain, A. (2008) Medicinal Chemistry, Chemotherapy: Antiseptics and Disinfectants. Unpublished report. Hamdard Nagar, New Delhi.
- Irwin, P. (1996). To clean up environmental spill, know your medium. *Electrical World* 37-40.
- Jenkins, D., Richard, M. and Daigger, G. (1993) Manual on the Causes and Control of Activated Sludge Bulking and Foaming, 2nd ed. Boca Raton: Lewis Publishers
- Jones, L. (1988) *Corrosion and water Technology*. Tulsa: OGCI publications.
- Jones, M., Herd, T., and Christie, H. (1989) Resistance of *Pseudomonas aeruginosa* to Amphoteric and Quaternary ammonium biocides. *Microbios* 9, 255–264.
- Kaeck, A. and Egli, T. (2001) Isolation and characterization of a *Pseudomonas putida* strain able to grow with trimethyl- 1,2-dihydroxy-propyl-ammonium as sole source of carbon, energy and nitrogen, *Syst. Appl. Microbiol.* 24 252–261.

REFERENCES

- Kaecha, A., Vallotton, N. and Eglia, T., (2005) Isolation and characterization of heterotrophic bacteria able to grow aerobically with quaternary ammonium alcohols as sole source of carbon and nitrogen, *Journal Systemic and Applied Microbiology* (28) 230-241
- Kahng , H., Malinverni, J., Majko, M. And Kukor, J. (2001) Genetic and functional analysis of the *tbc* operons for catabolism of alkyl- and chloroaromatic compounds in *Burkholderia* sp. Strain JS 150. *Appl Environ Microbiol* 67: 4805-4816
- Kastner, M., Streibich, S., Beyrer, M., Richnow, H. And Fritsche, W. (1999) Formation of bound residues during microbial degradation of (¹⁴C) anthracene in soil. *Appl Environ Microbiol* 65: 1834-1842.
- Klug, E. and Fresenius, Z. (1987) *Anal. Chem.* 329 490.
- Koike, M., Iida, K. and Matsuo, T. (1969). Electron microscopic studies on mode of action of polymyxin. *J. Bacteriol.* 97:448-452.
- Kouma, H., Shibata, S. and Nagata, T. (2003). Preparation of quaternary ammonium salts having antibacterial and antifungal activity. *Jpn. Kokai Tokkyo Koho patent JP* 2003137706.
- Krahn, M., and Stein, E. (1998). Assessing Exposure of Marine Biota and Habitats to Petroleum Compounds. *Analytical Chemistry News & Features* 186-192.
- Kroon, A., Pomper, M. and van Ginkel, G. (1994) Metabolism of dodecyldimethylamine of *Pseudomonas* MA3. *Applied Microbiology and Biotechnology* 42, 134–139.
- Langsrud, S. and Sundheim, G. (1997). Factors contributing to the survival of poultry associated *Pseudomonas* spp. exposed to a quaternary ammonium compound. *J. Appl. Microbiol.* 82:705–712.
- Langsrud, S., Sundheim, G. and Strahsen R. (2003). Intrinsic and acquired resistance to quaternary ammonium compounds in food-related *Pseudomonas* spp. *J. Appl. Microbiol.* 95:874–882.

REFERENCES

- Larson, R. and Perry, R. (1981) Use of the electrolytic respirometer to measure biodegradation in natural waters. *Water Res.* 15:697-702.
- Larson, R. and Vashon, R. (1983) Adsorption and biodegradation of carionic surfactants in laboratory and environmental systems, *Dev .Ind. Microbiol.* 24: 425-434.
- Leahy, J. and Colwell, R. (1990) Microbial degradation of hydrocarbons in the environment. *Microbiol Rev* 54: 305-315
- Lee, M., Novotny, M. and Bartle, K. (1981) *Analytical Chemistry of Polycyclic Aromatic Compounds*. Academic Press, New York.
- Lewis, M. and Wee, V. (1983) Aquatic safety assessment for cationic surfactants. *Environ. Toxicol. Chem.* 2:105-118.
- Liffourrena, A., Lo'pez, F., Salvano, M., Domenech, C and Lucchesi, G. (2008) Degradation of tetradecyltrimethylammonium by *Pseudomonas putida* A ATCC 12633 restricted by accumulation of trimethylamine is alleviated by addition of Al^{3+} ions. *Journal of Applied Microbiology* 104. pp 396–402.
- López-lópez, A., Pujalte, M., Benlloch, S., Mata-roig, M., Rosselló-mora, R., Garay, E. and Rodríguez-valera, F. (2002) : *Thalassospira lucentensis* gen. nov., sp. nov., a new marine member of the α -*Proteobacteria*. *Int. J. Syst. Evol. Microbiol.*, 52, 1277-1283.
- Lovley, D. (2003). "Cleaning up with genomics: applying molecular biology to bioremediation". *Nature Reviews. Microbiology*. 1 (1): 35 – 44
- MacGregor, D. and Eiliker, P. (1958) A comparison of some properties of strains of *Pseudomonas aeruginosa* sensitive and resistant to quaternary ammonium compounds. *Can. J. Microbiol.* 4:499-503.
- McFarlane, J., Bostick, D. and Luo, H. (2002) "Characterization and Modelling of Produced Water," presented at the 2002 Ground Water Protection Council Produced Water Conference, Colorado Springs, CO.

- Madigan, M., Martinko, J. and Parker, J. (2004). *Brock Biology of Microorganisms* (10th ed.). Lippincott Williams & Wilkins.
- Mallampati, S., Bhavesh mehta, S., Manish, J., Leena, K., Venkatrama Krishna, S., Shaik, B. and Gadde ramachandraiah, P. (2007) Bioaccumulation of heavy metals in some commercial fishes and crabs of the Gulf of Cambay, India. *Current Science* Vol. 92, no. 11, 1489-1491.
- Mansour, S. and Sidky, M. (2002) Ecotoxicological studies. 3. Heavy metals contaminating water and fish from Fayoum Governorate, *Egypt. Food Chem.* 78, pp. 15–22
- Marcotte, L., Barbeau, J., and Lafleur, M. (2005). "Permeability and thermodynamics study of quaternary ammonium surfactants--phosphocholine vesicle system." *Journal of Colloid and Interface Science* 292(1): 219-227.
- Masuda, F., Machida, S. and Kanno, M. (1976) Studies on the biodegradability of some cationic surfactants. pp. 129-138. In *Proceedings of the 7th international congress on surface active agents*, Moscow.
- McBain, A., Ledder, R., Moore, L., Catrenich, C. and Gilbert, P. (2004) Effects of quaternary-ammonium-based formulations on bacterial community dynamics and antimicrobial susceptibility. *Appl. Environ. Microbiol.* 70:3449–3456.
- Meagher, R. (2000). "Phytoremediation of toxic elemental and organic pollutants". *Current Opinion in Plant Biology* 3 (2): 153–162.
- Mehandjiyska, L. (1995). "Microbiological analysis of activated sludge in municipal wastewater treatment plant at 'kremikovtzi' holding." *Journal of Culture Collections*, 1, 18–22.

REFERENCES

- Melville, F. and Burchett, M. (2002) Genetic variation in *Avicennia marina* in three estuaries of Sydney (Australia) and implications for rehabilitation and management. *Marine Pollution Bulletin* 44, pp. 469–479
- Michalik, B. (2008). NORM impacts on the environment: an approach to complete environmental risk assessment using the example of areas contaminated due to mining activity. *Appl Radiat Isot.* 66(11):1661-1665.
- Nagai, K., Murata, T., Ohta, S., Zenda, H., Ohnishi, M. and Hyashi, T. (2003) Two different mechanisms are involved in the extremely high-level benzalkonium chloride resistance of a *Pseudomonas fluorescens* strain. *Microbiol. Immunol.* 47:709–715.
- Nakano, M. and Zuber, P. (1998) "Anaerobic growth of a "strict aerobe" (*Bacillus subtilis*)". *Annu Rev Microbiol* 52: 165–90.
- Nguyen, P., van Ginkel, C. and Plugge, C (2008) Anaerobic degradation of long-chain alkylamines by a denitrifying *Pseudomonas stutzeri* FEMS Microbiol Ecol 66 136–142.
- Nielsen, J., and Nielsen, P.(2002). “Quantification of functional groups in activated sludge by microautoradiography.” *Water Sci. Technol.*, 46(1–2), 389–395.
- Nielsen, P., Thomsen, T., and Nielsen, J. (2004). “Bacterial composition of activated sludge - Importance for floc and sludge properties.” *Water Sci. Technol.*, 49(10), 51–58.
- Nishihara, T., Okamoto, T., Nishiyama, N. (2000) Biodegradation of didecyldimethylammonium chloride by *Pseudomonas fluorescens* TN4 isolated from activated sludge, *Appl. Environ. Microbiol.* 88 641–647.

REFERENCES

- Nishihara, T., Yano, M., Kato, K. and Takasaki, A. (1998) Neutralizing effect of sodium laurate on the bactericidal action of a quaternary ammonium disinfectant against *Staphylococcus aureus*. *Biocontrol Science* 3, 1-5.
- Nishiyama, N. and Nishihara, T. (2002). Biodegradation of dodecyltrimethylammonium bromide by *Pseudomonas fluorescens* F7 and F2 isolated from activated sludge. *Microbes and Environment*, 17: No 4, pp 164-169
- Nishiyama, N., Toshima, Y. and Ikeda, Y. (1995) Biodegradation of alkyltrimethylammonium salts in activated sludge. *Chemosphere* 30, 593–603.
- Nishikawa, M., Nakajima, K., Tatsuno, M., Kasuya, F., Igarashi, K., Fukui, M. and Tsuchihashi, H. (1994). The analysis of cocaine and its metabolites by liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry (LC/APCI-MS). *Forensic Sci Int* 1; 66:149.
- Nishikawa, M., Tatsuno, M., Katagi, M. and Tsuchihashi, H. (1996) Simultaneous determination of illicit drugs in human urine by liquid chromatography-mass spectrometry, *J. Anal. Toxicol.* 20: 281-286
- OECD 301D, (1992) Guidelines for Testing of Chemicals. Closed Bottle Test. Organisation of Economic Cooperation and Development, Paris.
- Orszulik, S. (2008) Environmental technology in the oil industry, 2nd ed. Springer publishers.
- Parales, R., Bruce, N., Schmid, A. and Wackett, L. (2002) Biodegradation, biotransformation and biocatalysis. *Appl Environ Microbiol* 68:4699-4709.
- Parr, A., Smith, M., Beveridge, C., Kerr, A., Cowling, M. and Hodgkiess, T. (1998) Advanced Materials in Optics and Electronics 8, 187–193.

REFERENCES

- Patrauchan. M. and Oriel, P. (2003) Degradation of benzyldimethylalkylammonium chloride by *Aeromonas hydrophila* sp. K. *Journal of Applied Microbiology* 2003 94,266-272.
- Pitter, P. and Svitalkova, J.(1961) Synthetic surfactants in wastewaters.III..Biodegradation of cationic agents in laboratory models of activated sludge tanks. *Sb. Vys. Sk. Chem.-Technol.* 5(2):25-42.
- Pitter, P., Hollerova, J. and Simanova J. (1976) Biodegradability of cationic surfactants. *Surf. Cong.* 4:82-92.
- Prenafeta Boldú, F., Kuhn, A., Luykx, D., Anke, H., van Groenestijn, J. and de Bont, J. (2001) Isolation and characterisation of fungi growing on volatile aromatic hydrocarbons as their sole carbon and energy source. *Mycol. Res.* 105: 477–484
- Preston, A. (1983) Dialkyldimethylammonium halides as wood preservatives. *Journal of the American Oil Chemistry Society* 60, 567-570.
- Raffaelli, A. and Bruins, A. (1991). Factors Affecting the Ionization Efficiency of Quarternary Ammonium Compounds in Electrospray/ Ionspray Mass Spectrometry. *Rapid Commun. MassSpectrom.*, 5, 269–275.
- Rashed, M., Ozand, P., Bucknall, M. and Little, D. (1995) Diagnosis of inborn errors of metabolism from blood spots by acylcarnitines and amino acids profiling using automated electrospray tandem mass spectrometry. *Pediatr Res.*; 38:324–31.
- Ray, P. and Engelhardt, R. (1992) *Produced Water: Technological/Environmental Issues and Solutions*, Plenum Publishing Corporation. NY
- Reis, J. (1996) *Environmental Control in Petroleum Engineering*. Gulf Publishing Company London.

REFERENCES

- Resnick, S., Lee, K. and Gibson, D. (1996) Diverse reactions catalysed by naphthalene dioxygenase from *pseudomonas ap.* Strain NCIB 9816. *J Ind Microbiol Biotechnol* 7:438-456
- Ruiz Cruz, J. (1979) Pollution of natural waters by synthetic detergents XVI. Influence of surfactant concentration and bacterial acclimation on biodegradation of cationic surfactants in river water *Grasas Aceites* 30:293-299.
- Russell, A., Hammond, S. and Morgan, J. (1986) Bacterial resistance to antiseptics and disinfectants. *J. Hosp Infect* 7, 213–225.
- Russell, A., (1992a). Factors influencing the efficacy of antimicrobial agents. In: Russell, A., Hugo, W., Ayliffe, G. (Eds.), *Disinfection. Preservation and Sterilization*. Blackwell Scientific Publications, p. 89-113.
- Russell, A. (1992b). Bacterial sensitivity and resistance. In: Russell, A., Hugo, W., Ayliffe, G. (Eds.). *Disinfection, Preservation and Sterilization*. Blackwell Scientific Publications, p. 211-229.
- Russell, A. and Chopra, I. (1996) *Understanding Antibacterial Action and Resistance*, 2nd edn. Chichester, England: Ellis Horwood.
- Russell, A. (2002). Mechanisms of antimicrobial action of antiseptics and disinfectants: an increasingly important area of investigation. *J. Antimicrob. Chemother.* 49:597–599.
- Ryan, K. and Ray, C. (2004). *Sherrie Medical Microbiology* (4th ed.). McGraw Hill. pp. 232–233.
- Schlegel, H. (1985) *General microbiology* 6th Edition Cambridge University Press, New York.

REFERENCES

- Seki, R., Kamimura, R., Mitsunashi, S., Shirai, J., Nitta, M. and Takatori, K. (1993) Intensified effects of the cationic surfactant disinfectant alkalinized with NaOH. *Japan Bulletin of Animal Hygiene* 36, 7-18.
- Servin J. A, Herbold, C., Skophammer, R., and Lake, J. (2008). "Evidence excluding the root of the tree of life from the actinobacteria". *Mol. Biol. Evol.* 25 (1): 1–4.
- Sharifi-Yazdi, M., Azimi, C., and Khalili, M. (2001). "Isolation and identification of bacteria present in the activated sludge unit, in the treatment of industrial waste water." *Iranian J. Publ. Health*, 30(3–4),91–94.
- Slager, L.(1992). Environmental Aspects of Produced Water Discharges from Oil and Gas Production on the Dutch Continental Shelf. Part III. Environmental Effects. Free University of Amsterdam.
- Singh, R., Gupta, N., Singh, S., Singh, A., Suman, R. and Annie, K. (2002) Toxicity of ionic and non-ionic surfactants to six microbes found in Agra, India. *Bull Environ Contam Toxicol*; 69:265–70.
- Somerville, H., Bennet, D., Davenport, J., and Holt, M. (1987) Environmental Effect of Produced Water from North Sea Oil Operations. *Marine Pollution Bulletin*, 18, No 10,549-558.
- Spain, J., Pritchard, P. and Bourquin, A. (1980). Effects of adaptation on biodegradation rates in sediment/water cores from estuarine and freshwater environments. *Appl. Environ. Microbiol.* 40:726-734
- Spain, J. and Van Veld, P. (1983). Adaptation of natural microbial communities to degradation of xenobiotic compounds: effects of concentration, exposure time, inoculum, and chemical structure. *Appl. Environ. Microbiol.* 45:428-435.
- Stephenson, M. (1992) Components of Produced Water: A Compilation of Industry Studies. *JPT*, pp 548-603.

REFERENCES

- Stromgren, T., Sorstrom, S., Schou, L., Kaarstad, I., Aunaas, T., Brakstad, O. and Johansen, O. (1995) Acute Toxic Effects of Produced Water in Relation to Chemical Composition and Dispersion. *Marine Environmental Research*, 40, No 2, pp 147-169.
- Sullivan, D. (1983) Biodegradation of a cationic surfactant in activated sludge, *Water Res.*, 17, 1145-1151.
- Sundheim, G., Hagtvedt, T. and Dainty, R. (1992). Resistance of meat associated staphylococci to a quaternary ammonium compound. *Food Microbiol* 9:161–167.
- Swannell, R., Lee, K. and McDonagh, M. (1996). Field evaluations of marine oil spill bioremediation. *Microbiol. Rev.* 60:342–365.
- Swisher, R. (1970) “Surfactant Biodegradation”, *Surfactant Science Series*; Marcel Dekker: New York, Vol. 3.
- Swisher, R. (1987) “Surfactant Biodegradation”, *Surfactant Science Series*, Marcel Dekker, New York, vol. 18.
- Szumala, A. and Pernak, J. (1986) The natural and laboratory resistance of Gram-negative rods to quaternary ammonium chlorides whit alkylthiomethyl radical. *Pharmazie* 41, 521.
- Tabata, A., Nagamune, H., Maeda, T., Murakami, K., Miyake, Y., and Kourai, H (2003) Correlation between Resistance of *Pseudomonas aeruginosa* to Quaternary Ammonium Compounds and Expression of Outer Membrane Protein OprR Antimicrobial Agents And Chemotherapy July, p. 2093–2099.
- Takenaka, S., Tonoki, T., Taira, K., Murakami, S. and Aoki, K. (2007) Adaptation of *Pseudomonas* sp. Strain 7-6 to Quaternary Ammonium Compounds and Their Degradation via Dual Pathways. *Appl. Env. Microbiology* (6) 73.1797-1802.

REFERENCES

- Takasaki, A., Hashida, T., Fujiwara, S., Kato, K. and Nishihara, T. (1994a) Bactericidal action of a quaternary ammonium disinfectant, didecyldimethylammonium chloride, against *Staphylococcus aureus*. *Japanese Journal of Toxicology and Environmental Health* 40, 344-350.
- Takasaki, A., Hashida, T., Kato, K., Moriyama, T. and Nishihara, T. (1994b) Action of a quaternary ammonium disinfectant on cell membrane of *Staphylococcus aureus*. *Japanese Journal of Toxicology and Environmental Health* 40, 520- 526.
- Tomlinson, E., Brown, M. and Davis, S. (1977). Effect of colloidal association on the measured activity of alkylbenzyldimethylammonium chlorides against *Pseudomonas aeruginosa*. *J. Med. Chem.* 20:1277–1282.
- Topping, B. and Waters, J. (1982) Monitoring of cationic surfactants in sewage treatment plants, *Tenside Surf. Det.*, 19,164-169.
- Tubbing, D. and Admiraal, W. (1991) Inhibition of bacterial and phytoplanktonic metabolic activity in the lower River Rhine by ditallowdimethylammonium chloride. *Applied and Environmental Microbiology* 57, 3612–3622.
- UKOOA (1998) Chemical Discharges to the Marine Environment. Available from: <http://www.ukooa.co.uk/issues/1998report/v0000913.htm>
- Ulas, T. (2009). Fate and Effect of Quaternary Ammonium Compounds in Biological Systems. A PhD Thesis Presented to the School of Civil and Environmental Engineering Georgia Institute of Technology
- Utsunomiya, A., Watanuki, T., Matsushita, K., Nishina, M. and Tomita, I. (1997) Assessment of the toxicity of linear alkylbenzene sulfonate and quaternary alkylammonium chloride by measuring ¹³C-glycerol in *Dunaliella* sp. *Chemosphere*;35:2479– 90.

REFERENCES

- Utvik, T. (2003) "Composition and Characteristics of Produced Water in the North Sea, "Produced Water Workshop, Aberdeen, Scotland.
- Valle's, B., Riva, M., Sappera, C., Canela, M. and Griful, E. (2000) Toxicity values for cationic surfactants, *Tenside Surf. Det.* 37 290–295.
- van Ginkel, G. and Kolvenbach, M. (1991) Relations between structure of QACs salts and their biodegradability. *Chemosphere* 28:281–289.
- van Ginkel, G. (1995) Biodegradability of cationic surfactants. In *Biodegradability of Surfactants*. (edited by Karsaand, D. and Porter, M.), Blackie Academic & Professional, Chapman and Hall. London.
- van Ginkel, G. (1996) Complete degradation of xenophobic surfactants by consortia of aerobic microorganisms. *Biodegradation* 7, 151–164.
- van Ginkel, G., van Dijk, J. and Kroon, A. (1992) Metabolism of hexadecyltrimethylammonium chloride in *Pseudomonas* strain B1. *Applied and Environmental Microbiology* 17, 1145–1151.
- Van Hamme, J., Wong, E., Dettman, H., Gray, M., and Pickard, M. (2003) Dibenzyl sulphide metabolism by white rot fungi. *Appl Environ Microbiol* 69: 1320-1324
- Ventullo, R. and Larson, R. (1986). Adaptation of aquatic microbial communities to quaternary ammonium compounds. *Appl. Environ. Microbiol.* 51:356–361.
- Verbina, N. (1973) The influence of quaternary ammonium compounds on microorganism and their practical use. *Prikladnaja Biokhimiya i Mikrobiologija* 9, 575–578.
- Versteeg, D. and Shorter, S. (1992) Effect of organic carbon on the uptake and toxicity of quaternary ammonium compounds to the fathead minnow, *Pimephales promelas*. *Environ. Toxicol. Chem.* 11:571–580.

REFERENCES

- Vik, A. and Bakke, S. (1992) Norwegian Approaches to Environmental Impact Assessment of Corrosion Inhibitors used Offshore. Aquateam, Norwegian Water Technology Centre, Oslo, Norway.
- Wackett, L. and Hershberger, C. (2001) Biocatalysis and biodegradation: microbial transformation of organic compounds. ASM Press, Washington, DC. pp 36-69
- Wagner, M., and Loy, A. (2002). "Bacterial community composition and function in sewage treatment systems." *Curr. Opin. Biotechnol.*, 13, 218–227.
- Waters, J. and Kupfer, W. (1976). The determination of cationic surfactants in the presence of anionic surfactant in biodegradation test liquors. *Anal. Chim. Acta* 85:241-251
- Watling, H. (1983). Accumulation of seven metals by *Crassostrea gigas*, *C. margaritacea*, *Perna perna* and *Chromytilus meridionalis*. *Bull. Environ. Contam. Toxicol.* 30, pp. 213–320.
- White, D. and McDermott, P. (2001). Biocides, drug resistance and microbial evolution. *Curr. Opin. Microbiol.* 4:313–317.
- Wilson, M. McNab, R. and Henderson, B. (2002) Bacterial Disease Mechanisms: *An introduction to cellular microbiology*. Cambridge University Press. pp. 51-52
- Xu, R., Obbard, J., and Tay, E (2003). World Journal of Microbiology & Biotechnology, 19, 719-725
- Ying, G. (2006) Fate, behavior and effects of surfactants and their degradation products in the environment, *Environment International* 32, 417 – 431, York, NY.
- Yuwono, T (2005) Metabolism of betaine as a carbon source by an osmotolerant bacterium isolated from the weed rhizosphere *World Journal of Microbiology & Biotechnology* 21: 69–73

REFERENCES

- Zhang, Q., Simpson, J. and Aboleneen, H. (1997) A specific method for the measurement of tacrolimus in human whole blood by liquid chromatography / tandem mass spectrometry. *Thera Drug Monit.* 19:470–6.

APPENDIX

APPENDIX A

A.1: BDHAC RECOVERY ANALYSIS (CHAPTER 2)

Sample with 5mg BDHAC in 100ml solution of growth medium + standard and with absorbance value of 1.591

5mg Quat in 100ml solution in 250ml flask (concentration Quat = 0.05mg/ml)

50ml was removed for analysis

Amount of Quat used analysis = $50 \times 0.05 = 2.5\text{mg}$

50ml of sample was then extracted with 50ml DCM

Extract into 50ml DCM (contains 2.5mg Quat if no degradation)

25ml DCM (containing 1.25mg Quat) was blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as 10.219ug/ml (measured from calibration standards)

In 50ml seawater we have 510.95ug or 0.51095mg Quat

Originally we would have had 1.25mg or 1250ug Quat in 25ml sample if no degradation.

Therefore the percentage recovery = $510.95/1250 \times 100 = 40.88\%$

A.2: BDHAC RECOVERY ANALYSIS (CHAPTER 3 Section A)

Sample with 40mg BDHAC in 10.8ml and an absorbance value of 0.673 diluted 1 in 10 with chloroform

40mg Quat in 10.8ml solution in the flask (concentration Quat = 3.704mg/ml)

3.2ml removed therefore 7.6ml remaining

Amount of Quat remaining 28.147mg (concentration Quat = 3.704mg/ml)

Extract into 10ml DCM (contains 28.147mg Quat if no degradation)

1ml DCM (containing 2.814mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as 4.399ug/ml (measured from calibration standards)

$4.399\text{ug/ml} \times 10 \text{ [dilution factor]} = 43.99\text{ug/ml}$

In 50ml seawater we have 2199.5ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have $(2199.5\text{ug} \times 10) = 21995\text{ug}$ Quat if used original 10ml solution of DCM

Originally we would have had 28147ug Quat in 7.6ml sample if no degradation.

Therefore the percentage recovery = $(21995/28147 \times 100) = 78.14\%$

Sample with 30mg BDHAC in 10.6ml of solution and absorbance value of 0.458 diluted 1 in 10 with chloroform

30mg Quat in 10.6ml solution in the flask (concentration Quat = 2.830mg/ml)

3.2ml removed therefore 7.4ml remaining

Amount of Quat remaining 20.943mg (concentration Quat = 2.830mg/ml)

Extract into 10ml DCM (contains 20.943mg Quat if no degradation)

1ml DCM (containing 2.0943mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as 2.994ug/ml (measured from calibration standards)

Concentration x dilution factor of 10 = 29.94ug/ml

In 50ml seawater we have 1497ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have $(1497\text{ug} \times 10) = 14970\text{ug}$ Quat if used original 10ml solution of DCM

Originally we would have had 20943.36ug Quat in 7.4ml sample if no degradation.

Therefore the percentage recovery = $(14970/20943.36 \times 100) = 71.47\%$

Sample with 25mg BDHAC in 10.5ml of solution and absorbance value 0.380 diluted 1 in 10 with chloroform

25mg Quat in 10.5ml solution in the flask (concentration Quat = 2.3809mg/ml)

3.2ml removed therefore 7.3ml remaining

Amount of Quat remaining 17.381mg (concentration Quat = 2.3809mg/ml)

Extract into 10ml DCM (contains 17.381mg Quat if no degradation)

1ml DCM (containing 1.7381mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as 2.484ug/ml (measured from calibration standards)

Concentration x dilution factor of 10 = 24.837ug/ml

In 50ml seawater we have 1241.85ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have $(1241.85\text{ug} \times 10) = 12418.50\text{ug}$ Quat if used original 10ml solution of DCM

Originally we would have had 17381.12ug Quat in 7.3ml sample if no degradation.

Therefore the percentage recovery = $(12418.50/17381.12 \times 100) = 71.45\%$

Sample with 20mg BDHAC in 10.4ml of solution and absorbance value of 0.210 diluted 1 in 10 with chloroform

20mg Quat in 10.4ml solution in the flask (concentration Quat = 1.923mg/ml)

3.2ml removed therefore 7.2ml remaining

Amount of Quat remaining 13.846mg (concentration Quat = 1.923mg/ml)

Extract into 10ml DCM (contains 13.846mg Quat if no degradation)

1ml DCM (containing 1.385mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as 1.373ug/ml (measured from calibration standards)

Concentration x dilution factor = $1.373 \times 10 = 13.73 \text{ug/ml}$

In 50ml seawater we have 686.5ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have $(686.5 \text{ug} \times 10) = 6865 \text{ug}$ Quat if used original 10ml solution of DCM

Originally we would have had 13846.1ug Quat in 7.2ml sample if no degradation.

Therefore the percentage recovery = $(6865/13846.1 \times 100) = 49.56\%$

Sample with 10mg BDHAC in 10.2ml of solution and absorbance value of 1.450

10mg Quat in 10.2ml solution in the flask (concentration Quat = 0.980mg/ml)

3.2ml removed therefore 7.0ml remaining

Amount of Quat remaining 6.863mg (concentration Quat = 0.980mg/ml)

Extract into 10ml DCM (contains 6.863 mg Quat if no degradation)

1ml DCM (containing 0.6863mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as 9.477ug/ml (measured from calibration standards)

In 50ml seawater we have 473.855ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have $(473.855 \text{ug} \times 10) = 4738.55 \text{ug}$ Quat if used original 10ml solution of DCM

Originally we would have had 6863ug Quat in 7.0ml sample if no degradation.

Therefore the percentage recovery = $(4738.55/6863 \times 100) = 69.05\%$

Sample with 5mg BDHAC in 10.1ml of solution and absorbance value of 0.595

5mg Quat in 10.1ml solution in the flask (concentration Quat = 0.495mg/ml)

3.2ml removed therefore 6.9ml remaining

Amount of Quat remaining 3.416mg (concentration Quat = 0.495mg/ml)

Extract into 10ml DCM (contains 3.416mg Quat if no degradation)

1ml DCM (containing 0.3416mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as 3.889ug/ml (measured from calibration standards)

In 50ml seawater we have 194.45ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have $(194.45\text{ug} \times 10) = 1944.5\text{ug}$ Quat if used original 10ml solution of DCM

Originally we would have had 3416ug Quat in 6.9ml sample if no degradation.

Therefore the percentage recovery = $(1944.5/3416 \times 100) = 56.92\%$

Sample with 0.5mg BDHAC in 10.01ml solution of sea water + std and with absorbance value of 0.035

0.5mg Quat in 10.01ml solution in the flask (concentration Quat = 0.04995mg/ml)

3.2ml removed therefore 6.81ml remaining

Amount of Quat remaining 0.340mg (concentration Quat = 0.04995mg/ml)

Extract into 10ml DCM (contains 0.340mg Quat if no degradation)

1ml DCM (containing 0.0340mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as 0.229ug/ml (measured from calibration standards)

In 50ml seawater we have 11.45ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have $(11.45\text{ug} \times 10) = 114.5\text{ug}$ Quat if used original 10ml solution of DCM

Originally we would have had 340.16ug Quat in 6.81ml sample if no degradation

Therefore the percentage recovery = $114.5/340.16 \times 100 = 33.63\%$

A.3: BDHAC RECOVERY ANALYSIS (CHAPTER 3 Section B)

Sample with 400mg of BDHAC in 100ml and an absorbance value of 0.652 diluted 1 in 10 with chloroform

But from recent calibration curve $Y = 0.1446x + 0.1135$

Y = absorbance ; X = concentration in ug/ml

400mg Quat in 100ml solution in the flask (concentration Quat = 4mg/ml)

5ml removed for DBA analysis

Amount used for DBA analysis $5\text{ml} \times 4\text{mg/ml} = 20\text{mg}$

Extract into 10ml DCM (contains 20 mg Quat if no degradation)

1ml DCM (containing 2mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as 3.7241ug/ml (measured from calibration standards)

$3.7241\text{ug/ml} \times 10$ [dilution factor] = 37.241ug/ml

In 50ml seawater we have 1862.05ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have $(1862.05\text{ug} \times 10)$
 $= 18620.5\text{ug}$ (18.621mg) Quat if used original 10ml solution of DCM

Originally we would have had 20mg Quat in 5ml sample.

Therefore the percentage recovery = $(18.621/20 \times 100) = 93.10\%$

Sample with 200mg of BDHAC in 100ml and an absorbance value of 0.369

200mg Quat in 100ml solution in the flask (concentration Quat = 2mg/ml)

5ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $5\text{ml} \times 2\text{mg/ml} = 10\text{mg}$

Extract into 10ml DCM (contains 10mg Quat if no degradation)

1ml DCM (containing 1mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as 1.7669ug/ml (measured from calibration standards)

$1.7669\text{ug/ml} \times 10$ [dilution factor] = 17.669ug/ml

In 50ml seawater we have 883.45ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have $(883.45\text{ug} \times 10)$
 $= 8834.5\text{ug}$ (8.835mg) Quat if used original 10ml solution of DCM

Originally we would have had 10mg Quat in 5ml sample.

Therefore the percentage recovery = $(8.835/10 \times 100) = 88.35\%$

A.4: BDHAC RECOVERY ANALYSIS (CHAPTER 3 Section C)

Sample with 10mg of BDHAC in 100ml and an absorbance value of 0.330

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

10mg Quat in 100ml solution in the flask (concentration Quat = 0.1mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 0.1\text{mg/ml} = 0.2\text{mg}$

Extract into 10ml DCM (contains 0.2mg Quat if no degradation)

Concentration from absorbance measured as 2.161ug/ml (measured from calibration standards)

In 50ml seawater we have 108.06ug Quat

108.06ug (0.1081mg) Quat if used original 10ml solution of DCM

Originally we would have had 0.2mg Quat in 10ml sample.

Therefore the percentage recovery = $(0.1081/0.2 \times 100) = 54.05\%$

Sample with 25 mg of BDHAC in 100ml and an absorbance value of 0.885

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

25mg Quat in 100ml solution in the flask (concentration Quat = 0.25mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 0.25\text{mg/ml} = 0.5\text{mg}$

Extract into 10ml DCM (contains 0.5mg Quat if no degradation)

Concentration from absorbance measured as 5.796ug/ml (measured from calibration standards)

In 50ml seawater we have 289.78ug Quat

289.78ug (0.290mg) Quat if used original 10ml solution of DCM

Originally we would have had 0.5mg Quat in 10ml sample.

Therefore the percentage recovery = $(0.290/0.5 \times 100) = 57.96\%$

Sample with 50mg of BDHAC in 100ml and an absorbance value of 1.625

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

50mg Quat in 100ml solution in the flask (concentration Quat = 0.5mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 0.5\text{mg/ml} = 1.0\text{mg}$

Extract into 10ml DCM (contains 1.0mg Quat if no degradation)

Concentration from absorbance measured as 10.642ug/ml (measured from calibration standards)

In 50ml seawater we have 532.09ug Quat

532.09ug (0.532mg) Quat if used original 10ml solution of DCM

Originally we would have had 1.0mg Quat in 10ml sample.

Therefore the percentage recovery = $(0.532/1.0 \times 100) = 53.20\%$

Sample with 100mg of BDHAC in 100ml and an absorbance value of 2.890

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

100mg Quat in 100ml solution in the flask (concentration Quat = 1.0mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 1.0\text{mg/ml} = 2.0\text{mg}$

Extract into 10ml DCM (contains 2.0mg Quat if no degradation)

Concentration from absorbance measured as 18.926ug/ml (measured from calibration standards)

In 50ml seawater we have 946.30ug Quat

946.30ug (0.946mg) Quat if used original 10ml solution of DCM

Originally we would have had 2.0mg Quat in 10ml sample.

Therefore the percentage recovery = $(0.946/2.0 \times 100) = 47.30\%$

APPENDIX B**B.1 QUANTIFICATION OF RESIDUAL BDHAC BY COLORIMETRIC DYE
BINDING ASSAY (CDBA) (CHAPTER 2)****Control Sample**

5mg Quat in 100ml solution in 250ml flask (concentration Quat = 0.05mg/ml)

50ml was removed for ESI/MS-MS and CDBA analysis

Amount of Quat used for both analysis = $50 \times 0.05 = 2.5\text{mg}$

50ml of sample was then extracted with 50ml DCM

Extract into 50ml DCM (contains 2.5mg Quat if no degradation)

25ml DCM (containing 1.25mg Quat) was blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as **8.400**ug/ml (measured from calibration standards)

In 50ml seawater we have 420ug or 0.42mg Quat

Originally we would have had 1.25mg Quat in 25ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.42/1.25 \times 100) = 66.40\%$

1.25 mg of Quat in the flask has been degraded by 66.40% (0.83mg lost)

Therefore 0.42mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is 42.21%

Therefore amount that would have been recovered if no absorption

$= 0.42/42.21 \times 100 = 0.995\text{mg}$

Starting amount of Quat was 1.25mg, therefore

% degradation = $100 - (0.995/1.25 \times 100) = 20.4\%$

Sample from flask DP1

5mg Quat in 100ml solution in 250ml flask (concentration Quat = 0.05mg/ml)

50ml was removed for ESI/MS-MS and CDBA analysis

Amount of Quat used for both analysis = $50 \times 0.05 = 2.5\text{mg}$

50ml of sample was then extracted with 50ml DCM

Extract into 50ml DCM (contains 2.5mg Quat if no degradation)

25ml DCM (containing 1.25mg Quat) was blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as 5.2953ug/ml (measured from calibration standards)

In 50ml seawater we have 264.765ug or 0.2648mg Quat

Originally we would have had 1.25mg Quat in 25ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.2648/1.25 \times 100) = 78.82\%$

1.25 mg of Quat in the flask has been degraded by 78.82% (0.985mg lost)

Therefore 0.2648mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is 42.21%

Therefore amount that would have been recovered if no absorption

= $0.2648/42.21 \times 100 = 0.627\text{mg}$

Starting amount of Quat was 1.25mg, therefore

% degradation = $100 - (0.62734 / 1.25 \times 100) = 49.81\%$

Sample from flask DP2

5mg Quat in 100ml solution in 250ml flask (concentration Quat = 0.05mg/ml)

50ml was removed for ESI/MS-MS and CDBA analysis

Amount of Quat used for both analysis = $50 \times 0.05 = 2.5\text{mg}$

50ml of sample was then extracted with 50ml DCM

Extract into 50ml DCM (contains 2.5mg Quat if no degradation)

25ml DCM (containing 1.25mg Quat) was blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as **3.5733**ug/ml (measured from calibration standards)

In 50ml seawater we have 178.665ug or 0.178665mg Quat

Originally we would have had 1.25mg Quat in 25ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.179/1.25 \times 100) = 85.71\%$

1.25 mg of Quat in the flask has been degraded by 85.71% (1.071mg lost)

Therefore 0.1786mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is 42.21%

Therefore amount that would have been recovered if no absorption

= $0.1786/42.21 \times 100 = 0.4231$ mg

Starting amount of Quat was 1.25mg, therefore

% degradation = $100 - (0.42312 / 1.25 \times 100) = 66.15\%$

Sample from flask DP3

5mg Quat in 100ml solution in 250ml flask (concentration Quat = 0.05mg/ml)

50ml was removed for ESI/MS-MS and CDBA analysis

Amount of Quat used for both analysis = $50 \times 0.05 = 2.5$ mg

50ml of sample was then extracted with 50ml DCM

Extract into 50ml DCM (contains 2.5mg Quat if no degradation)

25ml DCM (containing 1.25mg Quat) was blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as **3.0408**ug/ml (measured from calibration standards)

In 50ml seawater we have 152.040ug or 0.15204mg Quat

Originally we would have had 1.25mg Quat in 25ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.1520/1.25 \times 100) = 87.84\%$

1.25 mg of Quat in the flask has been degraded by 87.84% (1.098mg lost)

Therefore 0.152mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is 42.21%

Therefore amount that would have been recovered if no absorption

$$= 0.152/42.21 \times 100 = 0.3601 \text{ mg}$$

Starting amount of Quat was 1.25mg, therefore

$$\% \text{ degradation} = 100 - (0.3601 / 1.25 \times 100) = 71.19\%$$

Sample from flask DP4

5mg Quat in 100ml solution in 250ml flask (concentration Quat = 0.05mg/ml)

50ml was removed for ESI/MS-MS and CDBA analysis

Amount of Quat used for both analysis = $50 \times 0.05 = 2.5\text{mg}$

50ml of sample was then extracted with 50ml DCM

Extract into 50ml DCM (contains 2.5mg Quat if no degradation)

25ml DCM (containing 1.25mg Quat) was blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as **6.0975**ug/ml (measured from calibration standards)

In 50ml seawater we have 304.876ug or 0.3049mg Quat

Originally we would have had 1.25mg Quat in 25ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.3049/1.25 \times 100) = 75.61\%$

1.25 mg of Quat in the flask has been degraded by 75.61% (0.945mg lost)

Therefore 0.3049mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is 42.21%

Therefore amount that would have been recovered if no absorption

$$= 0.3049/42.21 \times 100 = 0.7223 \text{ mg}$$

Starting amount of Quat was 1.25mg, therefore

$$\% \text{ degradation} = 100 - (0.7223 / 1.25 \times 100) = 42.22\%$$

Sample from flask DP5

5mg Quat in 100ml solution in 250ml flask (concentration Quat = 0.05mg/ml)

50ml was removed for ESI/MS-MS and CDBA analysis

Amount of Quat used for both analysis = $50 \times 0.05 = 2.5\text{mg}$

50ml of sample was then extracted with 50ml DCM

Extract into 50ml DCM (contains 2.5mg Quat if no degradation)

25ml DCM (containing 1.25mg Quat) was blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as **4.3755**ug/ml (measured from calibration standards)

In 50ml seawater we have 218.776ug or 0.2188mg Quat

Originally we would have had 1.25mg Quat in 25ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.2188/1.25 \times 100) = 82.50\%$

1.25 mg of Quat in the flask has been degraded by 82.50% (1.0313mg lost)

Therefore 0.2188mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is 42.21%

Therefore amount that would have been recovered if no absorption

= $0.2188/42.21 \times 100 = 0.5184\text{ mg}$

Starting amount of Quat was 1.25mg, therefore

% degradation = $100 - (0.5184/1.25 \times 100) = 58.53\%$

B.2 QUANTIFICATION OF RESIDUAL BDHAC BY ELECTROSPRAY IONISATION MASS SPECTROMETRY (ESI-MS/MS) (CHAPTER 2)

Control Sample

Peak Area [C16/C14] = 1.28652122

From the calibration Curve, the equation to the line is given as $y=0.0395x-0.05$

Therefore $1.28652122 = 0.0395x-0.05$

$1.33652122=0.0395x$

$X=33.83598\text{ug/ml}$ (Concentration of Quat)

This means that a Peak Area of 1.28652122 was obtained at a concentration of 33.83598 ug/ml 25ml will be $[33.83598\text{ug/ml} \times 25\text{ml}] = 845.8995\text{ug}$ (0.8459mg).

In the degradation experiment, 25ml of DCM extract contained 1.25mg of Quat,

% Degradation $= 100 - [(0.8459/1.25) \times 100] = 32.33\%$

Sample from flask DP1

Peak Area [C16/C14] = 0.463769238

From the calibration Curve, the equation to the line is given as $y=0.0395x-0.05$

Therefore $0.463769238 = 0.0395x-0.05$

$0.51376924=0.0395x$

$X=13.0068\text{ug/ml}$ (Concentration of Quat)

This means that a Peak Area of 0.463769238 was obtained at a concentration of 13.0068ug/ml 25ml will be $[13.0068\text{ug/ml} \times 25\text{ml}] = 325.1704\text{ug}$ (0.3252mg).

In the degradation experiment, 25ml of DCM extract contained 1.25mg of Quat,

% Degradation $= 100 - [(0.3252/1.25) \times 100] = 73.99\%$

Sample from flask DP2

Peak Area [C16/C14] = 0.925168309

From the calibration Curve, the equation to the line is given as $y=0.0395x-0.05$

Therefore $0.925168309 = 0.0395x-0.05$

$0.975168309=0.0395x$

$X=24.6878\text{ug/ml}$ (Concentration of Quat)

This means that a Peak Area of 0.925168309 was obtained at a concentration of 24.6878ug/ml 25ml will be $[24.6878\text{ug/ml} \times 25\text{ml}] = 617.1951\text{ug}$ (0.6172mg).

In the degradation experiment, 25ml of DCM extract contained 1.25mg of Quat,

% Degradation $= 100 - [(0.6172/1.25) \times 100] = 50.62\%$

Sample from flask DP3

Peak Area [C16/C14] = 0.241727737

From the calibration Curve, the equation to the line is given as $y=0.0395x-0.05$

Therefore $0.241727737 = 0.0395x-0.05$

$0.291727737=0.0395x$

$X=7.3855\text{ug/ml}$ (Concentration of Quat)

This means that a Peak Area of 0.241727737 was obtained at a concentration of 7.3855ug/ml 25ml will be $[7.3855\text{ug/ml} \times 25\text{ml}] = 184.6378\text{ug}$ (0.1846mg).

In the degradation experiment, 25ml of DCM extract contained 1.25mg of Quat,

% Degradation $= 100 - [(0.1846/1.25) \times 100] = 85.23\%$

Sample from flask DP4

Peak Area [C16/C14] = 0.325684674

From the calibration Curve, the equation to the line is given as $y=0.0395x-0.05$

Therefore $0.325684674 = 0.0395x-0.05$

$0.375684674=0.0395x$

$X=9.5110\text{ug/ml}$ (Concentration of Quat)

This means that a Peak Area of 0.325684674 was obtained at a concentration of 9.5110 ug/ml 25ml will be $[9.5110\text{ug/ml} \times 25\text{ml}] = 237.7751\text{ug}$ (0.2378mg).

In the degradation experiment, 25ml of DCM extract contained 1.25mg of Quat,

$$\% \text{ Degradation} = 100 - [(0.2378/1.25) \times 100] = 80.98\%$$

Sample from flask DP5

Peak Area [C16/C14] = 0.860795996

From the calibration Curve, the equation to the line is given as $y = 0.0395x - 0.05$

Therefore $0.860795996 = 0.0395x - 0.05$

$$0.910795996 = 0.0395x$$

$X = 23.0581\text{ug/ml}$ (Concentration of Quat)

This means that a Peak Area of 0.860795996 was obtained at a concentration of 23.0581ug/ml 25ml will be $[23.0581\text{ug/ml} \times 25\text{ml}] = 576.4531\text{ug}$ (0.5765mg).

In the degradation experiment, 25ml of DCM extract contained 1.25mg of Quat,

$$\% \text{ Degradation} = 100 - [(0.5765/1.25) \times 100] = 53.88\%$$

APPENDIX C**C.1: QUANTIFICATION OF RESIDUAL BDHAC AFTER BIODEGRADATION****EXPERIMENT BY COLORIMETRIC DYE BINDING ASSAY (CDBA)****(CHAPTER 3 Section A)****STRAIN G**

Sample in batch G with 40mg of BDHAC in 10.8ml and an absorbance value of 1.916

40mg Quat in 10.8ml solution in the flask (concentration Quat =3.704mg/ml)

3.2ml removed therefore 7.6ml remaining

Amount of Quat remaining 28.147mg (concentration Quat =3.704mg/ml)

Extract into 10ml DCM (contains 28.147mg Quat if no degradation)

1ml DCM (containing 2.8147mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as 12.523ug/ml (measured from calibration standards)

In 50ml seawater we have 626.15ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have 6.2615mg (626.15ugx10) Quat if used original 10ml solution of DCM

Originally we would have had 28.147mg Quat in 7.6ml sample if no degradation.

Therefore the percentage degradation = $100 - (6.2615/28.147 \times 100) = 77.76\%$

28.147mg of Quat in the test tube has been degraded by 77.76% (21.8873mg lost)

Therefore 6.2599mg Quat have not been degraded, i.e. have been recovered

But recovery for this amount of Quat is 78.22%

Therefore amount that would have been recovered if no absorption

= $6.2599/78.22 \times 100 = 8.003 \text{ mg}$

Starting amount of Quat was 28.1472mg, therefore

% degradation = $100 - (8.003 / 28.147 \times 100) = 71.57\%$

Sample with 30mg BDHAC in 10.6ml of solution and absorbance value of 0.998

30mg Quat in 10.6ml solution in the flask (concentration Quat = 2.8302mg/ml)

3.2ml removed therefore 7.4ml remaining

Amount of Quat remaining 20.94336mg (concentration Quat = 2.8302mg/ml)

Extract into 10ml DCM (contains 20.94336mg Quat if no degradation)

1ml DCM (containing 2.094336mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as 6.523ug/ml (measured from calibration standards)

In 50ml seawater we have 326.15ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have 3.2614mg (326.15ugx10) Quat if used original 10ml solution of DCM

Originally we would have had 20.9434mg Quat in 7.4ml sample if no degradation.

Therefore the percentage degradation = $100 - (3.2615/20.9434 \times 100) = 84.43\%$

20.94336mg of Quat in the test tube has been degraded by 84.43% (17.683mg lost)

Therefore 3.2609mg Quat have not been degraded, i.e. have been recovered

But recovery for this amount of Quat is 71.26%

Therefore amount that would have been recovered if no absorption

= $3.2609/71.26 \times 100 = 4.576 \text{ mg}$

Starting amount of Quat was 20.94336mg, therefore

% degradation = $100 - (4.576/20.9434 \times 100) = 78.15\%$

Sample with 25mg BDHAC in 10.5ml of solution and absorbance value of 0.475

25mg Quat in 10.5ml solution in the flask (concentration Quat = 2.381mg/ml)

3.2ml removed therefore 7.3ml remaining

Amount of Quat remaining 17.3811mg (concentration Quat = 2.381mg/ml)

Extract into 10ml DCM (contains 17.3811mg Quat if no degradation)

1ml DCM (containing 1.73811mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as 3.1046ug/ml (measured from calibration standards)

In 50ml seawater we have 155.23ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have 1.5523mg (155.23ugx10) Quat if used original 10ml solution of DCM

Originally we would have had 17.3811mg Quat in 7.3ml sample if no degradation.

Therefore the percentage degradation = $100 - (1.5523/17.3811 \times 100) = 91.07\%$

17.3811mg of Quat in the test tube has been degraded by 91.07% (15.829mg lost)

Therefore 1.5521mg Quat have not been degraded, i.e. have been recovered

But recovery for this amount of Quat is 72.26%

Therefore amount that would have been recovered if no absorption

= $1.5521/72.26 \times 100 = 2.1480$ mg

Starting amount of Quat was 17.3811mg, therefore

% degradation = $100 - (2.1480 / 17.3811 \times 100) = 87.64\%$

Sample with 20mg BDHAC in 10.4ml of solution and absorbance value of 0.13

20mg Quat in 10.4ml solution in the flask (concentration Quat = 1.923mg/ml)

3.2ml removed therefore 7.2ml remaining

Amount of Quat remaining 13.8461mg (concentration Quat = 1.923mg/ml)

Extract into 10ml DCM (contains 13.846mg Quat if no degradation)

1ml DCM (containing 1.3846mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as 0.8497ug/ml (measured from calibration standards)

In 50ml seawater we have 42.485ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have 0.42485mg

(42.485ug x10) Quat if used original 10ml solution of DCM

Originally we would have had 13.846mg Quat in 7.2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.42485/13.846 \times 100) = 96.93\%$

13.846mg of Quat in the test tube has been degraded by 96.93% (13.421mg lost)

Therefore 0.4251mg Quat have not been degraded, i.e. have been recovered

But recovery for this amount of Quat is 51.22%

Therefore amount that would have been recovered if no absorption

= $0.4251/51.22 \times 100 = 0.8299$ mg

Starting amount of Quat was 13.846mg, therefore

% degradation = $100 - (0.8299 / 13.846 \times 100) = 94\%$

Sample with 10mg BDHAC in 10.2ml of solution and absorbance value of 0.118

10mg Quat in 10.2ml solution in the flask (concentration Quat = 0.980mg/ml)

3.2ml removed therefore 7.0ml remaining

Amount of Quat remaining 6.8627mg (concentration Quat = 0.980mg/ml)

Extract into 10ml DCM (contains 6.8627 mg Quat if no degradation)

1ml DCM (containing 0.68627mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as 0.7712ug/ml (measured from calibration standards)

In 50ml seawater we have 38.56ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have 0.3856mg (38.56ugx10) Quat if used original 10ml solution of DCM

Originally we would have had 6.8627mg Quat in 7.0ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.3856/6.8627 \times 100) = 94.38\%$

6.8627mg of Quat in the test tube has been degraded by 94.38% (6.477mg lost)

Therefore 0.3857mg Quat have not been degraded, i.e. have been recovered

But recovery for this amount of Quat is 69.27%

Therefore amount that would have been recovered if no absorption

= $0.3857/69.27 \times 100 = 0.5568$ mg

Starting amount of Quat was 6.8627mg, therefore

$$\% \text{ degradation} = 100 - (0.5568 / 6.8627 \times 100) = 91.87\%$$

Sample with 5mg BDHAC in 10.1ml of solution and absorbance value of 0.048

5mg Quat in 10.1ml solution in the flask (concentration Quat = 0.495mg/ml)

3.2ml removed therefore 6.9ml remaining

Amount of Quat remaining 3.416mg (concentration Quat = 0.495mg/ml)

Extract into 10ml DCM (contains 3.416mg Quat if no degradation)

1ml DCM (containing 0.3416mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as 0.3137ug/ml (measured from calibration standards)

In 50ml seawater we have 15.686ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have 0.15686mg (15.686ug x 10) Quat if used original 10ml solution of DCM

Originally we would have had 3.416mg Quat in 6.9ml sample if no degradation.

$$\text{Therefore the percentage degradation} = 100 - (0.15686 / 3.416 \times 100) = 95.41\%$$

3.416mg of Quat in the test tube has been degraded by 95.41% (3.259mg lost)

Therefore 0.1568mg Quat have not been degraded, i.e. have been recovered

But recovery for this amount of Quat is 54.31%

Therefore amount that would have been recovered if no absorption

$$= 0.1568 / 54.31 \times 100 = 0.2887 \text{ mg}$$

Starting amount of Quat was 3.416mg, therefore

$$\% \text{ degradation} = 100 - (0.2887 / 3.416 \times 100) = 91.55\%$$

Sample with 0.5mg BDHAC in 10.01ml of solution and absorbance value of 0.034

0.5mg Quat in 10.01ml solution in the flask (concentration Quat = 0.04995mg/ml)

3.2ml removed therefore 6.81ml remaining

Amount of Quat remaining 0.3402mg (concentration Quat = 0.04995mg/ml)

Extract into 10ml DCM (contains 0.3402mg Quat if no degradation)

1ml DCM (containing 0.03402mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as 0.2222ug/ml (measured from calibration standards)

In 50ml seawater we have 11.111ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have 0.1111mg (11.111ugx10) Quat if used original 10ml solution of DCM

Originally we would have had 0.3402mg Quat in 6.81ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.1111/0.3402 \times 100) = 67.34\%$

0.3402mg of Quat in the test tube has been degraded by 67.34% (0.2291mg lost)

Therefore 0.1111mg Quat have not been degraded, i.e. have been recovered

But recovery for this amount of Quat is 40.03%

Therefore amount that would have been recovered if no absorption

= $0.1111/40.03 \times 100 = 0.2775 \text{ mg}$

Starting amount of Quat was 0.34016mg, therefore

% degradation = $100 - (0.2775 / 0.34016 \times 100) = 18.42\%$

APPENDIX D

D.1 QUANTIFICATION OF RESIDUAL BDHAC AFTER BIODEGRADATION
EXPERIMENT BY COLORIMETRIC DYE BINDING ASSAY (CDBA)
(CHAPTER 3 Section B)

Sporosarcina sp.

Sample with 200mg of BDHAC in 100ml of growth media during 28 days of incubation.

Day 0 (absorbance= 1.926, concentration from calibration curve = 12.535ug/ml)

200mg Quat in 100ml solution in 250ml flask (concentration Quat = 2 mg/ml)

5ml was removed for DBA analysis

Amount of used for DBA analysis = $5.0 \times 2 = 10$ mg

Extract into 10ml DCM (contains 10mg Quat if no degradation)

1ml DCM (containing 1mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as **12.535ug/ml** (measured from calibration standards)

In 50ml seawater we have 626.75ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have 6.2675mg (463ugx10) Quat if used original 10ml solution of DCM

Originally we would have had 10mg Quat in 5ml sample if no degradation.

Therefore the percentage degradation = $100 - (6.2675/10 \times 100) = 37.33\%$

10mg of Quat has been degraded by 37.33% (3.733mg lost)

Therefore 6.2675mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is 88.35%

Therefore amount that would have been recovered if no absorption

= $6.2675/88.35 \times 100 = 7.0939$ mg

Day 7 (absorbance= 1.450, concentration from calibration curve = 9.243ug/ml)

200mg Quat in 100ml solution in 250ml flask (concentration Quat = 2mg/ml)

5ml was removed for DBA analysis

Amount of used for DBA analysis = $5.0 \times 2 = 10\text{mg}$

Extract into 10ml DCM (contains 10mg Quat if no degradation)

1ml DCM (containing 1mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as **9.243ug/ml** (measured from calibration standards)

In 50ml seawater we have 462.135ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have 4.62135mg (462.135ug \times 10) Quat if used original 10ml solution of DCM

Originally we would have had 10mg Quat in 5ml sample if no degradation.

Therefore the percentage degradation = $100 - (4.62135/10 \times 100) = 53.79\%$

10mg of Quat has been degraded by 53.79% (5.379mg lost)

Therefore 4.62135mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is 88.35%

Therefore amount that would have been recovered if no absorption

= $4.62135/88.35 \times 100 = 5.2307\text{mg}$

Day 14 (absorbance= 1.506, concentration from calibration curve = 9.630ug/ml)

200mg Quat in 100ml solution in 250ml flask (concentration Quat = 2mg/ml)

5ml was removed for DBA analysis

Amount of used for DBA analysis = $5.0 \times 2 = 10\text{mg}$

Extract into 10ml DCM (contains 20mg Quat if no degradation)

1ml DCM (containing 1mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as **9.630 ug/ml** (measured from calibration standards)

In 50ml seawater we have 481.5ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have 4.815mg (481.5ugx10) Quat if used original 10ml solution of DCM

Originally we would have had 10mg Quat in 5ml sample if no degradation.

Therefore the percentage degradation = $100 - (4.815/10 \times 100) = 51.85\%$

10mg of Quat has been degraded by 51.85% (5.185mg lost)

Therefore 4.815mg Quat have not been degraded, i.e. have been recovered

But recovery for this amount of Quat is 88.35%

Therefore amount that would have been recovered if no absorption

= $4.815/88.35 \times 100 = 5.4499\text{mg}$

Day 21 (absorbance= 0.257, concentration from calibration curve = 0.992ug/ml)

200mg Quat in 100ml solution in 250ml flask (concentration Quat = 2mg/ml)

5ml was removed for DBA analysis

Amount of used for DBA analysis = $5.0 \times 2 = 10\text{mg}$

Extract into 10ml DCM (contains 20mg Quat if no degradation)

1ml DCM (containing 1mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as **0.992ug/ml** (measured from calibration standards)

In 50ml seawater we have 49.62ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have 0.4962mg (49.62ugx10) Quat if used original 10ml solution of DCM

Originally we would have had 10mg Quat in 5ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.4962/10 \times 100) = 95.04\%$

10mg of Quat has been degraded by 95.04% (9.504mg lost)

Therefore 0.4962mg Quat have not been degraded, i.e. have been recovered

But recovery for this amount of Quat is 88.35%

Therefore amount that would have been recovered if no absorption

$$= 0.4962/88.35 \times 100 = 0.5616\text{mg}$$

Day 28 (absorbance= 0.248, concentration from calibration curve = 0.930 ug/ml)

200mg Quat in 100ml solution in 250ml flask (concentration Quat = 2mg/ml)

5ml was removed for DBA analysis

Amount of used for DBA analysis = $5.0 \times 2 = 10\text{mg}$

Extract into 10ml DCM (contains 20mg Quat if no degradation)

1ml DCM (containing 1mg Quat) blown to dryness and redissolved in 50ml seawater

Concentration from absorbance measured as **0.930** ug/ml (measured from calibration standards)

In 50ml seawater we have **46.51**ug Quat

But this is 1 from 10ml DCM, therefore in 50ml seawater we would have 0.4651mg (**46.51**ug \times 10) Quat if used original 10ml solution of DCM

Originally we would have had 10mg Quat in 5ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.4651/10 \times 100) = 95.35\%$

10mg of Quat has been degraded by 95.35% (9.535mg lost)

Therefore 0.4651mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat 88.35%

Therefore amount that would have been recovered if no absorption

$$= 0.4651/88.35 \times 100 = 0.5264\text{mg}$$

APPENDIX E

**E.1: QUANTIFICATION OF RESIDUAL BDHAC BY COLORIMETRIC DYE
BINDING ASSAY (CDBA) AFTER BIODEGRADATION EXPERIMENT
(CHAPTER 3 Section C)**

Thalassospira sp.

Sample with 10mg BDHAC in 100ml of media and incubated at 25⁰C for 120 hours

0 hrs (absorbance=0.226, concentration from calibration curve = 1.480ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

10mg Quat in 100ml solution in the flask (concentration Quat = 0.1mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis 2mlx 0.1mg/ml= 0.2mg

Extract into 10ml DCM (contains 0.2mg Quat if no degradation)

Concentration from absorbance measured as **1.480ug/ml** (measured from calibration standards)

In 50ml seawater we have 74.00ug Quat

74.00ug (0.074mg) Quat if used original 10ml solution of DCM

Originally we would have had 0.2mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.0740/0.2 \times 100) = 63\%$

0.2mg of Quat in the flask has been degraded by 63% (0.126mg lost)

Therefore 0.074mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **56.50%**

Therefore amount that would have been recovered if no absorption

= $0.074/56.50 \times 100 = 0.131 \text{ mg}$

8 hrs (absorbance=0.212, concentration from calibration curve =1.388ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

10mg Quat in 100ml solution in the flask (concentration Quat = 0.1mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 0.1\text{mg/ml} = 0.2\text{mg}$

Extract into 10ml DCM (contains 0.2mg Quat if no degradation)

Concentration from absorbance measured as **1.388** ug/ml (measured from calibration standards)

In 50ml seawater we have 69.417ug Quat

69.417ug (0.0694mg) Quat if used original 10ml solution of DCM

Originally we would have had 0.2mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.0694/0.2 \times 100) = 65.29\%$

0.2mg of Quat in the flask has been degraded by 65.29% (0.131mg lost)

Therefore 0.069mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **56.50%**

Therefore amount that would have been recovered if no absorption

$= 0.069/56.50 \times 100 = 0.123 \text{ mg}$

24 hrs (absorbance=0.167, concentration from calibration curve = 1.094ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

10mg Quat in 100ml solution in the flask (concentration Quat = 0.1mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 0.1\text{mg/ml} = 0.2\text{mg}$

Extract into 10ml DCM (contains 0.2mg Quat if no degradation)

Concentration from absorbance measured as **1.094**ug/ml (measured from calibration standards)

In 50ml seawater we have 54.682ug Quat

54.682ug (0.0547mg) Quat if used original 10ml solution of DCM

Originally we would have had 0.2mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.0547/0.2 \times 100) = 72.66\%$

0.2mg of Quat in the flask has been degraded by 72.66% (0.145mg lost)

Therefore 0.0547mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **56.50%**

Therefore amount that would have been recovered if no absorption

= $0.0547/56.50 \times 100 = 0.097\text{mg}$

48 hrs (absorbance=0.095, concentration from calibration curve = 0.622ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

10mg Quat in 100ml solution in the flask (concentration Quat = 0.1mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 0.1\text{mg/ml} = 0.2\text{mg}$

Extract into 10ml DCM (contains 0.2mg Quat if no degradation)

Concentration from absorbance measured as **0.622**ug/ml (measured from calibration standards)

In 50ml seawater we have 31.107ug Quat

31.107ug (0.031107mg) Quat if used original 10ml solution of DCM

Originally we would have had 0.2mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.031107/0.2 \times 100) = 84.45\%$

0.2mg of Quat in the flask has been degraded by 84.45% (0.169mg lost)

Therefore 0.031mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **56.50%**

Therefore amount that would have been recovered if no absorption

$$= 0.031/56.50 \times 100 = 0.055 \text{ mg}$$

72 hrs (absorbance=0.063, concentration from calibration curve = 0.413 ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

10mg Quat in 100ml solution in the flask (concentration Quat = 0.1mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 0.1\text{mg/ml} = 0.2\text{mg}$

Extract into 10ml DCM (contains 0.2mg Quat if no degradation)

Concentration from absorbance measured as **0.413 ug/ml** (measured from calibration standards)

In 50ml seawater we have 20.630ug Quat

20.650ug (0.02063mg) Quat if used original 10ml solution of DCM

Originally we would have had 0.2mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.02063/0.2 \times 100) = 89.69\%$

0.2mg of Quat in the flask has been degraded by 89.69% (0.179mg lost)

Therefore 0.021mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **56.50%**

Therefore amount that would have been recovered if no absorption

$$= 0.021/56.50 \times 100 = 0.036\text{mg}$$

120 hrs (absorbance=0.055, concentration from calibration curve = 0.360ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

10mg Quat in 100ml solution in the flask (concentration Quat = 0.1mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 0.1\text{mg/ml} = 0.2\text{mg}$

Extract into 10ml DCM (contains 0.2mg Quat if no degradation)

Concentration from absorbance measured as **0.360ug/ml** (measured from calibration standards)

In 50ml seawater we have 18.009ug Quat

18.009ug (0.018mg) Quat if used original 10ml solution of DCM

Originally we would have had 0.2mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.018/0.2 \times 100) = 91\%$

0.2mg of Quat in the flask has been degraded by 91% (0.182mg lost)

Therefore 0.018mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **56.50%**

Therefore amount that would have been recovered if no absorption

= $0.018/56.50 \times 100 = 0.032 \text{ mg}$

25mg/100ml

Considering Strain J in a sample with 25mg BDHAC in 100ml of media and incubated at 25°C for 0, 8, 24, 48, 72 and 120 hours

0 hrs (absorbance=0.246, concentration from calibration curve = 1.611ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

25mg Quat in 100ml solution in the flask (concentration Quat = 0.25mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 0.25\text{mg/ml} = 0.5\text{mg}$

Extract into 10ml DCM (contains 0.5mg Quat if no degradation)

Concentration from absorbance measured as **1.611ug/ml** (measured from calibration standards)

In 50ml seawater we have 80.550ug Quat

80.550ug (0.081mg) Quat if used original 10ml solution of DCM

Originally we would have had 0.5mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.081/0.5 \times 100) = 83.89\%$

0.5mg of Quat in the flask has been degraded by 83.89% (0.419mg lost)

Therefore 0.081mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **58.88%**

Therefore amount that would have been recovered if no absorption

= $0.081/58.88 \times 100 = 0.137 \text{ mg}$

8 hrs (absorbance=0.216, concentration from calibration curve = 1.415ug/ml)

But from recent calibration curve $Y = 0.1527x$

sY= absorbance ; X = concentration in ug/ml

25mg Quat in 100ml solution in the flask (concentration Quat = 0.25mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 0.25\text{mg/ml} = 0.5\text{mg}$

Extract into 10ml DCM (contains 0.5mg Quat if no degradation)

Concentration from absorbance measured as **1.415ug/ml** (measured from calibration standards)

In 50ml seawater we have 70.75ug Quat

70.750ug (0.07075mg) Quat if used original 10ml solution of DCM

Originally we would have had 0.5mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.07075/0.5 \times 100) = 85.85\%$

0.5mg of Quat in the flask has been degraded by 85.85% (0.429mg lost)

Therefore 0.071mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **58.88%**

Therefore amount that would have been recovered if no absorption

= $0.071/58.88 \times 100 = 0.120\text{mg}$

24 hrs (absorbance=0.169, concentration from calibration curve = 1.107ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

25mg Quat in 100ml solution in the flask (concentration Quat = 0.25mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 0.25\text{mg/ml} = 0.5\text{mg}$

Extract into 10ml DCM (contains 0.5mg Quat if no degradation)

Concentration from absorbance measured as **1.107ug/ml** (measured from calibration standards)

In 50ml seawater we have 55.350ug Quat

55.350ug (0.055mg) Quat if used original 10ml solution of DCM

Originally we would have had 0.5mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.055/0.5 \times 100) = 88.93\%$

0.5mg of Quat in the flask has been degraded by 88.93% (0.445mg lost)

Therefore 0.055mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **58.88%**

Therefore amount that would have been recovered if no absorption

$= 0.055/58.88 \times 100 = 0.094 \text{ mg}$

48 hrs (absorbance=0.127, concentration from calibration curve = 0.832ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

25mg Quat in 100ml solution in the flask (concentration Quat = 0.25mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 0.25\text{mg/ml} = 0.5\text{mg}$

Extract into 10ml DCM (contains 0.5mg Quat if no degradation)

Concentration from absorbance measured as **0.832ug/ml** (measured from calibration standards)

In 50ml seawater we have 41.585ug Quat

41.585ug (0.042mg) Quat if used original 10ml solution of DCM

Originally we would have had 0.5mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.042/0.5 \times 100) = 91.68\%$

0.5mg of Quat in the flask has been degraded by 91.68% (0.458mg lost)

Therefore 0.042mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **58.88%**

Therefore amount that would have been recovered if no absorption

= $0.042/58.88 \times 100 = 0.071 \text{ mg}$

72 hrs (absorbance=0.112, concentration from calibration curve = 0.733ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

25mg Quat in 100ml solution in the flask (concentration Quat = 0.25mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 0.25\text{mg/ml} = 0.5\text{mg}$

Extract into 10ml DCM (contains 0.5mg Quat if no degradation)

Concentration from absorbance measured as **0.733ug/ml** (measured from calibration standards)

In 50ml seawater we have 36.673ug Quat

36.673ug (0.037mg) Quat if used original 10ml solution of DCM

Originally we would have had 0.5mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.037/0.5 \times 100) = 92.67\%$

0.5mg of Quat in the flask has been degraded by 92.67% (0.463mg lost)

Therefore 0.037mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **58.88%**

Therefore amount that would have been recovered if no absorption

$$= 0.037/58.88 \times 100 = 0.062 \text{ mg}$$

120 hrs (absorbance=0.088, concentration from calibration curve = 0.576ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

25mg Quat in 100ml solution in the flask (concentration Quat = 0.25mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 0.25\text{mg/ml} = 0.5\text{mg}$

Extract into 10ml DCM (contains 0.5mg Quat if no degradation)

Concentration from absorbance measured as **0.576ug/ml** (measured from calibration standards)

In 50ml seawater we have 28.815ug Quat

28.815ug (0.029mg) Quat if used original 10ml solution of DCM

Originally we would have had 0.5mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.029/0.5 \times 100) = 94.24\%$

0.5mg of Quat in the flask has been degraded by 94.24% (0.471mg lost)

Therefore 0.029mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **58.88%**

Therefore amount that would have been recovered if no absorption

$$= 0.029/58.88 \times 100 = 0.049\text{mg}$$

50mg/100ml

Considering Strain J in a sample with 50mg BDHAC in 100ml of media and incubated at 25°C for 0, 8, 24, 48, 72 and 120 hours

0 hrs (absorbance=0.351, concentration from calibration curve = 2.299ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

50mg Quat in 100ml solution in the flask (concentration Quat = 0.5mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 0.5\text{mg/ml} = 1.0\text{mg}$

Extract into 10ml DCM (contains 1.0mg Quat if no degradation)

Concentration from absorbance measured as **2.299**ug/ml (measured from calibration standards)

In 50ml seawater we have 114.931ug Quat

114.931ug (0.115mg) Quat if used original 10ml solution of DCM

Originally we would have had 1.0mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.115/1.0 \times 100) = 88.51\%$

1.0mg of Quat in the flask has been degraded by 88.51% (0.885mg lost)

Therefore 0.115mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **53.70%**

Therefore amount that would have been recovered if no absorption

$= 0.115/53.70 \times 100 = 0.214 \text{ mg}$

8 hrs (absorbance=0.331, concentration from calibration curve = 2.168ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

50mg Quat in 100ml solution in the flask (concentration Quat = 0.5mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 0.5\text{mg/ml} = 1.0\text{mg}$

Extract into 10ml DCM (contains 1.0mg Quat if no degradation)

Concentration from absorbance measured as **2.168**ug/ml (measured from calibration standards)

In 50ml seawater we have 108.382ug Quat

108.382ug (0.108mg) Quat if used original 10ml solution of DCM

Originally we would have had 1.0mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.108/1.0 \times 100) = 89.16\%$

1.0mg of Quat in the flask has been degraded by 89.16% (0.892mg lost)

Therefore 0.108mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **53.70%**

Therefore amount that would have been recovered if no absorption

= $0.108/53.70 \times 100 = 0.202 \text{ mg}$

24 hrs (absorbance= 0.269 concentration from calibration curve =1.762ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

50mg Quat in 100ml solution in the flask (concentration Quat = 0.5mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 0.5\text{mg/ml} = 1.0\text{mg}$

Extract into 10ml DCM (contains 1.0mg Quat if no degradation)

Concentration from absorbance measured as **1.762** ug/ml (measured from calibration standards)

In 50ml seawater we have 88.081ug Quat

88.081ug (0.088mg) Quat if used original 10ml solution of DCM

Originally we would have had 1.0mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.088/1.0 \times 100) = 91.19\%$

1.0mg of Quat in the flask has been degraded by 91.19% (0.912mg lost)

Therefore 0.088mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **53.70%**

Therefore amount that would have been recovered if no absorption

= $0.088/53.70 \times 100 = 0.164 \text{ mg}$

48 hrs (absorbance=0.131, concentration from calibration curve = 0.858ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

50mg Quat in 100ml solution in the flask (concentration Quat = 0.5mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 0.5\text{mg/ml} = 1.0\text{mg}$

Extract into 10ml DCM (contains 1.0mg Quat if no degradation)

Concentration from absorbance measured as **0.858ug/ml** (measured from calibration standards)

In 50ml seawater we have 42.896ug Quat

42.896ug (0.043mg) Quat if used original 10ml solution of DCM

Originally we would have had 1.0mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.043/1.0 \times 100) = 95.71\%$

1.0mg of Quat in the flask has been degraded by 95.71% (0.957mg lost)

Therefore 0.043mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **53.70%**

Therefore amount that would have been recovered if no absorption

$= 0.043/53.70 \times 100 = 0.080 \text{ mg}$

72 hrs (absorbance=0.125, concentration from calibration curve = 0.819ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

50mg Quat in 100ml solution in the flask (concentration Quat = 0.5mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 0.5\text{mg/ml} = 1.0\text{mg}$

Extract into 10ml DCM (contains 1.0mg Quat if no degradation)

Concentration from absorbance measured as **0.819ug/ml** (measured from calibration standards)

In 50ml seawater we have 40.930ug Quat

40.930ug (0.041mg) Quat if used original 10ml solution of DCM

Originally we would have had 1.0mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.041/1.0 \times 100) = 95.91\%$

1.0mg of Quat in the flask has been degraded by 95.91% (0.959mg lost)

Therefore 0.041mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **53.70%**

Therefore amount that would have been recovered if no absorption

= $0.041/53.70 \times 100 = 0.076\text{mg}$

120 hrs (absorbance=0.090, concentration from calibration curve = 0.589ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

50mg Quat in 100ml solution in the flask (concentration Quat = 0.5mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 0.5\text{mg/ml} = 1.0\text{mg}$

Extract into 10ml DCM (contains 1.0mg Quat if no degradation)

Concentration from absorbance measured as **0.589ug/ml** (measured from calibration standards)

In 50ml seawater we have 29.470ug Quat

29.470ug (0.029mg) Quat if used original 10ml solution of DCM

Originally we would have had 1.0mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.029/1.0 \times 100) = 97.05\%$

1.0mg of Quat in the flask has been degraded by 97.05% (0.971mg lost)

Therefore 0.030mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **53.70%**

Therefore amount that would have been recovered if no absorption

$$= 0.030/53.70 \times 100 = 0.055\text{mg}$$

100mg/100ml

Considering Strain J in a sample with 100mg BDHAC in 100ml of media and incubated at 25°C for 0, 8, 24, 48, 72 and 120 hours

0 hrs (absorbance=0.926, concentration from calibration curve = 6.064ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y = absorbance ; X = concentration in ug/ml

100mg Quat in 100ml solution in the flask (concentration Quat = 1mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 1\text{mg/ml} = 2.0\text{mg}$

Extract into 10ml DCM (contains 2.0mg Quat if no degradation)

Concentration from absorbance measured as **6.064ug/ml** (measured from calibration standards)

In 50ml seawater we have 303.209ug Quat

303.209ug (0.303mg) Quat if used original 10ml solution of DCM

Originally we would have had 2.0mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.303/2.0 \times 100) = 84.84\%$

2.0mg of Quat in the flask has been degraded by 84.84% (1.697mg lost)

Therefore 0.303mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **47.48%**

Therefore amount that would have been recovered if no absorption

$$= 0.303/47.48 \times 100 = 0.639\text{mg}$$

8 hrs (absorbance=0.650, concentration from calibration curve = 4.257ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y = absorbance ; X = concentration in ug/ml

100mg Quat in 100ml solution in the flask (concentration Quat = 1mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 1\text{mg/ml} = 2.0\text{mg}$

Extract into 10ml DCM (contains 2.0mg Quat if no degradation)

Concentration from absorbance measured as **4.257**ug/ml (measured from calibration standards)

In 50ml seawater we have 212.836ug Quat

212.836ug (0.213mg) Quat if used original 10ml solution of DCM

Originally we would have had 2.0mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.213/2.0 \times 100) = 89.36\%$

2.0mg of Quat in the flask has been degraded by 89.36% (1.787mg lost)

Therefore 0.213mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **47.48%**

Therefore amount that would have been recovered if no absorption

$= 0.213/47.48 \times 100 = 0.448\text{mg}$

24 hrs (absorbance=0.425, concentration from calibration curve = 2.783ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

100mg Quat in 100ml solution in the flask (concentration Quat = 1mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 1\text{mg/ml} = 2.0\text{mg}$

Extract into 10ml DCM (contains 2.0mg Quat if no degradation)

Concentration from absorbance measured as **2.783**ug/ml (measured from calibration standards)

In 50ml seawater we have 139.162ug Quat

139.162ug (0.139mg) Quat if used original 10ml solution of DCM

Originally we would have had 2.0mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.139/2.0 \times 100) = 93.04\%$

2.0mg of Quat in the flask has been degraded by 93.04% (1.861mg lost)

Therefore 0.139mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **47.48%**

Therefore amount that would have been recovered if no absorption

= $0.139/47.48 \times 100 = 0.280\text{mg}$

48 hrs (absorbance=0.23, concentration from calibration curve = 1.506ug/ml)

But from recent calibration curve $Y = 0.1527x$

$Y = \text{absorbance}$; $X = \text{concentration in ug/ml}$

100mg Quat in 100ml solution in the flask (concentration Quat = 1mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 1\text{mg/ml} = 2.0\text{mg}$

Extract into 10ml DCM (contains 2.0mg Quat if no degradation)

Concentration from absorbance measured as **1.506ug/ml** (measured from calibration standards)

In 50ml seawater we have 75.311ug Quat

75.311ug (0.075mg) Quat if used original 10ml solution of DCM

Originally we would have had 2.0mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.075/2.0 \times 100) = 96.23\%$

2.0mg of Quat in the flask has been degraded by 96.23% (1.925mg lost)

Therefore 0.075mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **47.48%**

Therefore amount that would have been recovered if no absorption

= $0.075/47.48 \times 100 = 0.159\text{mg}$

72 hrs (absorbance=0.128, concentration from calibration curve = 0.838ug/ml)

But from recent calibration curve $Y = 0.1527x$

Y= absorbance ; X = concentration in ug/ml

100mg Quat in 100ml solution in the flask (concentration Quat = 1mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 1\text{mg/ml} = 2.0\text{mg}$

Extract into 10ml DCM (contains 2.0mg Quat if no degradation)

Concentration from absorbance measured as **0.838**ug/ml (measured from calibration standards)

In 50ml seawater we have 41.912ug Quat

41.912ug (0.042mg) Quat if used original 10ml solution of DCM

Originally we would have had 2.0mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.042/2.0 \times 100) = 97.90\%$

2.0mg of Quat in the flask has been degraded by 97.90% (1.958mg lost)

Therefore 0.042mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **47.48%**

Therefore amount that would have been recovered if no absorption

$= 0.042/47.48 \times 100 = 0.088\text{mg}$

120 hrs (absorbance=0.099, concentration from calibration curve = 0.648ug/ml)

But from recent calibration curve Y= 0.1527x

Y= absorbance ; X = concentration in ug/ml

100mg Quat in 100ml solution in the flask (concentration Quat = 1mg/ml)

2ml of sample was removed for DBA analysis

Amount of Quat used for DBA analysis $2\text{ml} \times 1\text{mg/ml} = 2.0\text{mg}$

Extract into 10ml DCM (contains 2.0mg Quat if no degradation)

Concentration from absorbance measured as **0.648**ug/ml (measured from calibration standards)

In 50ml seawater we have 32.417ug Quat

32.417ug (0.032mg) Quat if used original 10ml solution of DCM

Originally we would have had 2.0mg Quat in 2ml sample if no degradation.

Therefore the percentage degradation = $100 - (0.032/2.0 \times 100) = 98.38\%$

2.0mg of Quat in the flask has been degraded by 98.38% (1.968mg lost)

Therefore 0.032mg Quat have not been degraded, ie have been recovered

But recovery for this amount of Quat is **47.48%**

Therefore amount that would have been recovered if no absorption

= $0.032 / \mathbf{47.48} \times 100 = 0.068\text{mg}$

**E.2 TABLES SHOWING OD₆₅₀, ABSORBANCE AND AMOUNT OF RESIDUAL
BDHAC FROM GROWTH AND BIODEGRADATION EXPERIMENTS IN
SECTION C OF CHAPTER 3**

Table 1. Optical Density Values Showing the growth of *Bacillus niabensis* at Different Concentrations of BDHAC

| Conc. of BDHAC added (mg/ml) | 0hrs | 8hrs | 24hrs | 48hrs | 72hrs | 120hrs |
|-------------------------------|------------|------------|-------------|------------|------------|------------|
| 0.1 | 0.082±0.07 | 0.086±0.03 | 0.094±0.02 | 0.199±0.01 | 0.216±0.02 | 0.317±0.07 |
| 0.25 | 0.081±0.07 | 0.090±0.01 | 0.101±0.02 | 0.211±0.04 | 0.311±0.02 | 0.418±0.04 |
| 0.5 | 0.084±0.01 | 0.095±0.06 | 0.115±0.02 | 0.246±0.03 | 0.337±0.03 | 0.560±0.01 |
| 1 | 0.093±0.04 | 0.123±0.03 | 0.318±0.06 | 0.417±0.02 | 0.635±0.01 | 0.913±0.04 |
| with 2ml of methanol | 0.083±0.02 | 0.088±0.01 | 0.090±0.08 | 0.211±0.01 | 0.309±0.05 | 0.422±0.05 |
| with no BDHAC and no methanol | 0.082±0.07 | 0.097±0.04 | 0.102±0.015 | 0.231±0.06 | 0.330±0.02 | 0.378±0.07 |

Table 2. Optical Density Values Showing the Growth of *Bacillus subtilis* at Different Concentrations of BDHAC

| Conc. of BDHAC added (mg/ml) | 0hrs | 8hrs | 24hrs | 48hrs | 72hrs | 120hrs |
|-------------------------------|------------|------------|------------|------------|------------|------------|
| 0.1 | 0.081±0.01 | 0.090±0.01 | 0.097±0.02 | 0.139±0.03 | 0.215±0.04 | 0.315±0.04 |
| 0.25 | 0.090±0.01 | 0.093±0.01 | 0.101±0.04 | 0.178±0.06 | 0.240±0.02 | 0.488±0.01 |
| 0.5 | 0.097±0.01 | 0.100±0.03 | 0.119±0.03 | 0.279±0.08 | 0.500±0.02 | 0.857±0.06 |
| 1 | 0.099±0.01 | 0.456±0.07 | 0.939±0.08 | 0.977±0.02 | 1.367±0.06 | 1.513±0.02 |
| with 2ml of methanol | 0.083±0.02 | 0.103±0.02 | 0.104±0.04 | 0.139±0.02 | 0.213±0.03 | 0.463±0.09 |
| with no BDHAC and no methanol | 0.083±0.03 | 0.102±0.05 | 0.105±0.08 | 0.175±0.05 | 0.285±0.05 | 0.461±0.13 |

Table 3 Optical Density Values Showing the Growth of *Sporosarcina sp.* at Different Concentrations of BDHAC

| Conc. of BDHAC added (mg/ml) | 0hrs | 8hrs | 24hrs | 48hrs | 72hrs | 120hrs |
|------------------------------|------------|------------|------------|------------|------------|------------|
| 0.1 | 0.083±0.07 | 0.085±0.06 | 0.097±0.03 | 0.197±0.01 | 0.219±0.06 | 0.338±0.03 |
| 0.25 | 0.094±0.06 | 0.096±0.05 | 0.096±0.03 | 0.220±0.02 | 0.319±0.07 | 0.432±0.06 |
| 0.5 | 0.099±0.01 | 0.107±0.07 | 0.137±0.01 | 0.349±0.07 | 0.540±0.01 | 0.839±0.08 |
| 1 | 0.099±0.01 | 0.132±0.01 | 0.896±0.01 | 0.990±0.01 | 1.354±0.04 | 1.404±0.04 |
| with 2ml of methanol | 0.085±0.04 | 0.107±0.01 | 0.109±0.06 | 0.128±0.04 | 0.217±0.08 | 0.366±0.07 |
| with no BDHAC and methanol | 0.085±0.01 | 0.104±0.01 | 0.103±0.03 | 0.191±0.06 | 0.217±0.08 | 0.378±0.05 |

Table 4. Optical Density Values Showing the Growth of *Thalassospira sp.* at different Concentrations of BDHAC

| Conc. of BDHAC added (mg/ml) | 0hrs | 8hrs | 24hrs | 48hrs | 72hrs | 120hrs |
|-------------------------------|------------|------------|------------|------------|------------|------------|
| 0.1 | 0.081±0.01 | 0.086±0.01 | 0.098±0.02 | 0.133±0.01 | 0.208±0.02 | 0.313±0.01 |
| 0.25 | 0.085±0.05 | 0.097±0.04 | 0.097±0.03 | 0.218±0.04 | 0.321±0.04 | 0.427±0.03 |
| 0.5 | 0.087±0.03 | 0.101±0.01 | 0.117±0.02 | 0.256±0.02 | 0.341±0.05 | 0.615±0.07 |
| 1 | 0.088±0.04 | 0.134±0.03 | 0.355±0.04 | 0.427±0.04 | 0.633±0.08 | 0.931±0.04 |
| with 2ml of methanol | 0.084±0.04 | 0.097±0.01 | 0.101±0.02 | 0.144±0.03 | 0.257±0.05 | 0.410±0.08 |
| with no BDHAC and no methanol | 0.089±0.09 | 0.094±0.01 | 0.100±0.03 | 0.117±0.01 | 0.256±0.06 | 0.323±0.01 |

Table 5. Absorbance Values at Different Concentrations of BDHAC with *Bacillus niabensis*

| Conc of BDHAC (mg/ml) | 0hrs | 8hrs | 24hrs | 48hrs | 72hrs | 120hrs |
|-------------------------------|------------|------------|------------|------------|------------|------------|
| 0.1 | 0.235±0.06 | 0.212±0.01 | 0.147±0.02 | 0.082±0.02 | 0.074±0.01 | 0.053±0.07 |
| 0.25 | 0.278±0.05 | 0.220±0.06 | 0.170±0.01 | 0.114±0.08 | 0.091±0.08 | 0.086±0.03 |
| 0.5 | 0.357±0.01 | 0.327±0.08 | 0.272±0.08 | 0.172±0.07 | 0.130±0.02 | 0.096±0.01 |
| 1 | 0.857±0.04 | 0.635±0.08 | 0.428±0.08 | 0.236±0.01 | 0.174±0.07 | 0.099±0.01 |
| with 2ml of methanol | 0.061±0.05 | 0.068±0.09 | 0.061±0.01 | 0.070±0.02 | 0.072±0.01 | 0.127±0.09 |
| with no BDHAC and no methanol | 0.040±0.01 | 0.024±0.04 | 0.036±0.04 | 0.013±0.03 | 0.044±0.06 | 0.025±0.06 |

Table 6. Amount of Residual BDHAC after BDHAC Degradation with *Bacillus niabensis*

| Conc of BDHAC (mg/ml) | Incubation Time (hrs) and Amount of Residual BDHAC (mg) | | | | | |
|-----------------------|---|------------|------------|------------|------------|------------|
| | 0 | 8 | 24 | 48 | 72 | 120 |
| 0.1 | 0.136±0.01 | 0.123±0.07 | 0.086±0.02 | 0.048±0.01 | 0.043±0.01 | 0.031±0.04 |
| 0.25 | 0.154±0.03 | 0.123±0.04 | 0.095±0.03 | 0.063±0.02 | 0.051±0.03 | 0.048±0.01 |
| 0.5 | 0.218±0.01 | 0.200±0.05 | 0.166±0.03 | 0.101±0.05 | 0.072±0.02 | 0.061±0.02 |
| 1 | 0.591±0.03 | 0.438±0.03 | 0.295±0.03 | 0.163±0.01 | 0.121±0.04 | 0.070±0.02 |

Table 7. Absorbance Values at Different Concentrations of BDHAC with *Bacillus subtilis*

| Conc of BDHAC (mg/ml) | 0hrs | 8hrs | 24hrs | 48hrs | 72hrs | 120hrs |
|-------------------------------|------------|-------------|------------|------------|------------|------------|
| 0.1 | 0.232±0.01 | 0.224±0.06 | 0.171±0.06 | 0.094±0.08 | 0.079±0.01 | 0.056±0.06 |
| 0.25 | 0.246±0.07 | 0.237±0.01 | 0.183±0.03 | 0.109±0.08 | 0.085±0.04 | 0.079±0.08 |
| 0.5 | 0.363±0.04 | 0.354±0.01 | 0.248±0.04 | 0.127±0.04 | 0.118±0.08 | 0.096±0.03 |
| 1 | 0.387±0.07 | 0.363±0.05 | 0.289±0.06 | 0.145±0.02 | 0.123±0.01 | 0.102±0.04 |
| with 2ml of methanol | 0.026±0.08 | 0.038±0.01 | 0.037±0.06 | 0.043±0.01 | 0.056±0.08 | 0.057±0.08 |
| with no BDHAC and no methanol | 0.023±0.08 | 0.024±0.012 | 0.023±0.01 | 0.028±0.01 | 0.044±0.01 | 0.083±0.33 |

Table 8. Amount of Residual BDHAC after BDHAC Degradation with *Bacillus subtilis*

| | Incubation Time (hrs) and Amount of Residual BDHAC (mg) | | | | | |
|-----------------------|---|------------|------------|------------|------------|------------|
| Conc of BDHAC (mg/ml) | 0 | 8 | 24 | 48 | 72 | 120 |
| 0.1 | 0.135±0.01 | 0.130±0.03 | 0.099±0.03 | 0.054±0.01 | 0.046±0.02 | 0.033±0.04 |
| 0.25 | 0.137±0.02 | 0.132±0.02 | 0.102±0.01 | 0.060±0.02 | 0.048±0.02 | 0.044±0.02 |
| 0.5 | 0.227±0.02 | 0.216±0.01 | 0.152±0.02 | 0.078±0.02 | 0.072±0.01 | 0.059±0.02 |
| 1 | 0.267±0.04 | 0.250±0.04 | 0.199±0.01 | 0.100±0.01 | 0.085±0.01 | 0.071±0.04 |

Table 9. Absorbance Values at Different Concentrations of BDHAC with *Sporosarcina sp.*

| Conc of BDHAC (mg/ml) | 0hrs | 8hrs | 24hrs | 48hrs | 72hrs | 120hrs |
|-------------------------------|------------|------------|------------|------------|------------|------------|
| 0.1 | 0.239±0.01 | 0.219±0.01 | 0.155±0.06 | 0.095±0.01 | 0.083±0.02 | 0.055±0.06 |
| 0.25 | 0.255±0.08 | 0.229±0.01 | 0.169±0.01 | 0.099±0.01 | 0.091±0.05 | 0.075±0.02 |
| 0.5 | 0.348±0.05 | 0.244±0.01 | 0.190±0.01 | 0.104±0.01 | 0.101±0.04 | 0.097±0.07 |
| 1 | 0.397±0.03 | 0.261±0.07 | 0.200±0.02 | 0.129±0.08 | 0.111±0.08 | 0.110±0.08 |
| with 2ml of methanol | 0.041±0.04 | 0.046±0.07 | 0.063±0.02 | 0.071±0.04 | 0.070±0.06 | 0.069±0.05 |
| with no BDHAC and no methanol | 0.026±0.09 | 0.025±0.03 | 0.028±0.01 | 0.039±0.02 | 0.038±0.09 | 0.039±0.01 |

Table 10. Amount of Residual BDHAC after BDHAC Degradation with *Sporosarcina sp.*

| Conc of BDHAC (mg/ml) | Incubation Time (hrs) and Amount of Residual BDHAC (mg) | | | | | |
|-----------------------|---|------------|------------|------------|------------|------------|
| | 0 | 8 | 24 | 48 | 72 | 120 |
| 0.1 | 0.139±0.02 | 0.127±0.02 | 0.090±0.01 | 0.055±0.03 | 0.048±0.01 | 0.032±0.03 |
| 0.25 | 0.142±0.04 | 0.127±0.03 | 0.094±0.02 | 0.056±0.01 | 0.050±0.03 | 0.042±0.01 |
| 0.5 | 0.212±0.03 | 0.149±0.02 | 0.116±0.02 | 0.064±0.01 | 0.062±0.02 | 0.059±0.04 |
| 1 | 0.274±0.01 | 0.180±0.01 | 0.138±0.02 | 0.089±0.03 | 0.076±0.02 | 0.076±0.06 |

Table 11. Absorbance Values at Different Concentrations of BDHAC with *Thalassospira sp.*

| Conc of BDHAC (mg/ml) | 0hrs | 8hrs | 24hrs | 48hrs | 72hrs | 120hrs |
|-------------------------------|------------|------------|------------|------------|------------|------------|
| 0.1 | 0.263±0.05 | 0.214±0.02 | 0.154±0.02 | 0.097±0.02 | 0.064±0.01 | 0.053±0.03 |
| 0.25 | 0.243±0.04 | 0.219±0.04 | 0.162±0.01 | 0.128±0.01 | 0.115±0.04 | 0.086±0.03 |
| 0.5 | 0.346±0.07 | 0.340±0.01 | 0.260±0.01 | 0.132±0.01 | 0.124±0.02 | 0.094±0.05 |
| 1 | 0.873±0.08 | 0.631±0.03 | 0.473±0.07 | 0.238±0.01 | 0.141±0.02 | 0.102±0.04 |
| with 2ml of methanol | 0.020±0.07 | 0.020±0.01 | 0.023±0.01 | 0.181±0.06 | 0.185±0.03 | 0.189±0.04 |
| with no BDHAC and no methanol | 0.037±0.02 | 0.038±0.01 | 0.041±0.04 | 0.028±0.02 | 0.033±0.04 | 0.063±0.04 |

Table 12. Amount of Residual BDHAC after BDHAC Degradation with *Thalassospira sp.*

| | Incubation Time (hrs) and Amount of Residual BDHAC (mg) | | | | | |
|-----------------------|---|------------|------------|------------|------------|------------|
| Conc of BDHAC (mg/ml) | 0 | 8 | 24 | 48 | 72 | 120 |
| 0.1 | 0.132±0.01 | 0.124±0.01 | 0.090±0.01 | 0.056±0.01 | 0.037±0.01 | 0.031±0.02 |
| 0.25 | 0.135±0.03 | 0.122±0.02 | 0.090±0.02 | 0.072±0.02 | 0.064±0.03 | 0.048±0.02 |
| 0.5 | 0.211±0.04 | 0.208±0.02 | 0.159±0.02 | 0.081±0.02 | 0.075±0.01 | 0.057±0.03 |
| 1 | 0.602±0.05 | 0.435±0.02 | 0.320±0.06 | 0.164±0.02 | 0.097±0.01 | 0.074±0.02 |